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ÁREA DE CIENCIA Y TECNOLOGÍA DE LOS ALIMENTOS

Improving the biotechnological quality of white wines from Rias Baixas PDO

Memoria presentada por

Marta Juega Rivera

para optar al grado de

Doctor en Ciencia y Tecnología de los Alimentos

Trabajo realizado bajo la dirección de

Dr. A.V Carrascosa y Dr. Adolfo J. Martínez-Rodríguez

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CERTIFICAN: Que el trabajo titulado: "**Improving the biotechnological quality of white wines from Rias Baixas PDO**", del que es autora Marta Juega Rivera, ha sido realizado en el Instituto de Investigación en Ciencias de la Alimentación (CIAL) (CSIC-UAM) bajo su dirección y cumple las condiciones exigidas para optar al grado de Doctor por la Universidad Autónoma de Madrid y, por tanto, autorizamos su presentación.

Y para que conste, firman el presente Certificado en Madrid a de Enero de 2014

Fdo. Dr. Alfonso V. Carrascosa

Fdo. Dr. Adolfo J. Martínez-Rodriguez

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INTRODUCTION

1. Wine and quality.

In Article 2 of title I of Law 24/2003 of July 10th, of Vine and Wine, wine is defined by the Spanish legislation as "natural food obtained exclusively by the complete or partial alcoholic fermentation of fresh, or not crushed, grape or must "(BOE n º 165, Friday July 11th, 2003, 27165).

According to the estimate of the OIV, Spain is one of the world's greater wine producers: first in the ranking by planted field, third in production (due to a lower yield than France and Italy), is the second largest exporter in terms of volume, although third in terms of value, and is the fifth consumer. Due to wine's importance in economic, social and environmental terms, and the importance of wine as a country's image abroad, this sector is of extraordinary relevance in Spain.

Despite the wide range of dynamism in the sector, domestic wine consumption in Spain still offers some troubling data, the averge consumption is estimated below 20 liters per person and year, positioning Spain at the bottom of Europe. This situation has resulted in an increasing interest in foreign markets and the gradual incorporation of quality improvement

The quality strategy seeks the control of processes to obtain a product, in this case wine, safely and reproducibly (Carrascosa, 2008). In the Wine Statute of 1970 (Law 25/1970), the system of appellations of origin was established. These Protected Designations of Origin (PDO), are considered as the geographical name of the region, area, place or locality used to designate a product with characteristics and differential qualities mainly caused to the natural environment and it's winemaking and aging. In this wine statute, a reference in wine quality was also done (B.O.E. nº 291, 5 de diciembre de 1970, 19816-19828).

In the current Law 24/2003, in particular in the Title II, appears the Origin Protection and Quality of Wines System as a system that will ensure it's first principle, the quality, and maintain the diversity of these wines. The most universally adequate tool for quality assurance is ISO standard 9000 (Anonymous, 2000). In this norm, the concept of neutral quality is handled, which isolated from subjective or commercial aspects, would define wine quality as the degree to which the natural characteristics of wine meet certain requirements previously set. These features may be what are known as variables in research, which allow to perform a continuous quality improvement based on objective and scientific criteria, and is the concept of quality triggers in the present study. In order to objectively assess the adequacy of wine characteristics to predetermined conditions, it is necessary to know exactly what one wants to do, something that not always happens with producers. Depending on whether variables studied are associated with safety, organoleptic characteristics, bromatological aspects or susceptibility to the winemaking process, we speak about hygienic quality, sensory quality, nutritional quality and technological quallity (Carrascosa, 2009). In the present study the improvement of the sensorial quality of white wines from Rias Baixas PDO, was discussed.

The living organisms used in this study, as a biotechnological tool for improving the quality of the white wines from Rías Baixas PDO, were no vines (*Vitis vinifera* var white Albariño or Caiño), but the microorganims performing the alcoholic and malolactic fermentation, respectively. They were the yeasts and lactic acid bacteria present in musts and wines studied. However, the oenological approach of this work makes it necessary to understand the socio-economic and scientific aspects of the wines used.

2. Albariño and Caíño white wines from Rias Baixas PDO.

Rías Baixas PDO, located in the northwest of Spain, is characteristic for having a remarkable maritime climate, which allows it to be the largest producer of the grape variety Albariño (Vilanova *et al.*, 2008). Originally, the Rias Baixas PDO was divided into 3 sub-regions: Val do Salnes, O Rosal and Condado do Tea. Later on, two more subzones: Soutomaior and Ribeira do Ulla, were included. Besides being the V*itis vinifera var* Albariño the main variety in the PDO, the varieties white Loureira, white Treixadura or Marques, and white Caiño variety, are also authorized (Anonymus, 2012). In 2012, the 177 wineries in this PDO produced 119, 836 HL of wine, from 4,048 hectares of vineyards, with a 95% of the harvest of Albariño grape and a 1% of white grape Caíño (Anonymous, 2013).

White grape varieties of Albariño and Caíño from Rías Baixas PDO are grown simultaneously in the subzones mentioned, in particular in O Rosal, from where the musts and wines used in this study were obtained.

Albariño white grape variety was chosen in this study since it is the most established and the most important variety in the Rias Baixas PDO. In the last chapter of the current thesis, where malolactic fermentation of Rias Baixas PDO wines is studied, the Caiño white variety was also used. This variety is considered one of the least studied of the PDO and the most ancient in Galicia, but from which a better progress in coming years is expected. In fact, nowadays is already used for the production of at least 64 wines of the Rias Baixas PDO (Santiago *et al.* 2007a), and is one of the most important emerging variety in this PDO.

The important possition of both white Caiño and Albariño varieties in the Spanish and international wine scene, would not be understood without the extraordinary work done by the group of viticulture from the Galician Biological Mission (CSIC). The group began in 1987, with the thesis of Dr. Martinez, who led Dr. Mantilla and started at the Institute of

Agrobiological Research (CSIC) in Santiago de Compostela, with the clear objective of the conservation of the wine heritage of northwestern of Spain, including Galicia and Asturias.

From 1989-1990 in the Galician Biological Mission led by Dr. Mantilla and of his working group, appeared the collection of Galician and Asturian vines that since 1987 had started collecting (Malvar, 2005; Martínez-Rodríguez *et al.*, 2009), and did not stop to increase, despite the early death of Mantilla in 1992. Nowadays, this collection is considered the biggest collection of vines in the CSIC.

Regarding the Albariño variety, the retrieval and cataloging work of strains was continued by clonal selection (Martinez et al, 2005), applying ampelographic methodology (Martinez *et al.*, 1993, Martinez *et al.*, 1999) and resistance to fungal attacks (Boso *et al*, 2004) developed by the group itself, which finally allowed the assignment of 11 clones of Albariño variety to Provedo Viveros S.A. With the objetive to start the process of certification of the material for further marketing and once certification process was concluded for five of them between 2007 and 2008, an agreement, which allows growers assigned to the PDO to have a five-year period to use exclusively two of the clones with a discount on the selling price, was signed up.

In relation to white Caiño variety, a similar study has recently been initiated by the group of Dr. Martinez. Current results of this study have helped to clarify that the white Caiño variety, which did not even appear itself in viticulture books in the late XIX century, or in the XX century (Marcilla, 1942), is a different variety (Santiago *et al.*, 2007b), than Albariño that produces very unique wines. In literature as early as 1772, it is mentioned that the main source of production of white Caiño is focused on the O Rosal valley in Galicia, in Rías Baixas PDO, where are around 60 Ha, and in Portugal, Vinho Verde region, where not more than 10 Ha have been dedicated to its cultivation (Robinson *et al.*, 2012).

White wines made from *Vitis vinifera* var Albariño are young with a remarkable aromatic intensity in which the aromas of white fruits like apple, pear and citrus predominate, often supplemented with very pleasant floral and herbaceous notes (Carballeira *et al*, 2001; Dieguez *et al*, 2003). Precisely, in terms of quality these are their most important sensorial characteristics, so this study is primarily focused on them pretending their improvement through the use of native or ecotypic wine yeast, isolated from Rias Baixas PDO must and wines and by post-fermentation treatments.

In addition, these are wines with a considerable structure and current acidity that gives freshness and youthfulness without being aggressive. Wines with long mouthfeel, and whose aromas in mouth are thin and fresh. They show high levels of total acidity around 7 g / L (expressed as tartaric) and an adjusted alcoholic content which usually ranges between 12 and 13% vol. Normally, these are dry wines with low levels of residual sugar (2 to 5 g / L) and dried extract values between 20 and 27 g/L. Although their volatile acidity is variable, is often found between 0.2 and 0.5 g / L (Martinez-Rodriguez *et al*., 2009).

Vitis vinifera var white Caiño, is a variety that has become virtually extinguished in Spain, and now is undergoing a period of recovery because is no longer be mistaken for Albariño. Its production is so limited that is only used in Galician white grape wines to blend. This variety gives low yields, because bunches and grapes are really small. It produces wines with a alcoholic content between 12 and 13 % vol., with thin and ripe aromas, very complex, displaying the typical characteristics of the variety (such as ripe tropical fruits and aromatic herbs) and the terroir, (minerality and earthy notes in retronasal and nose). It's high concentration of glycerol makes the wines in mounth plenty of flavours and high smoothless, with a good structure and body, resulting open, sweet and with a final mouthfeel that seems endless, with a depth and fleshiness that make the wine alive and very long. In addition, its

high acidity adds freshness and also makes a very good wine for aging. However, besides these tasting notes taken from published information from various sources, the scientific study of monovarietal white Caiño wines has not been addressed before, as far as it goes the knowledge of the person who sets out to write the current thesis.

3. Yeasts and wine sensorial quality.

The fermentation of grape must is a complex microbiological process that involves the interaction between yeasts, bacteria and filamentous fungi (Fleet, 2007; Fugelsang *et al*., 1997). Among yeasts, which play an important part in the winemaking process, it clear the central role of *Saccharomyces cerevisiae (S. cerevisiae)*, principal responsible of the alcoholic fermentation.

Traditionally, wine production was based on spontaneous fermentations from yeast found on grapes' surface and in winery environment. The use of commercial avaliable dried yeast strains to inoculate grape must have become an usual parctice in wineries to establish a high initial yeast population able to accomplish a controlled alcoholic fermentation (Nikolaou *et al*., 2006). Over the last few years, there has been an increasing trend towards the use of new locally selected yeast strains for monitoring must fermentations. These local yeasts are presumed more competitive than active dried yeast, since they are adapted to their local environments and local winemaking conditions (Vilanova *et al*., 2005; Melero, 1992; Querol *et al*., 1992; Degré *et al*., 1989), helping to mantain typical sensorial properties of regional wines; in fact, each strain of *S. cerevisiae* is able to produce different types and quantities of secondary compounds, which are determinant on the desirable aromatic characteristics of a wine (Pretorius, 2000, Regodón *et al.*, 1997), affecting to their final quality.

Nowadays, increasing competivive markets have forced winemakers to adapt their wines in order to meet changing consumer preferences and/or increase the singularity of their products, which means thet there is an actual interest in improving the final quality of wine and it's main sensorial descriptors: aroma, flavour and color.

3.1 Aroma and volatile profile in wines.

Wine aroma depends on the content of aromatic volatiles (Sanchez-Palomo *et al.*, 2007). Although it is widely considered to be a key aspect of quality, not all volatile compounds contribute equally to wine aroma; in fact, the contribution of a specific compound is related to it's odour perception threshold, which is defined as the lowest concentration that can be detected by smelling. The concentration of a specific compound/threshold ratio known as the ''odour activity value'' (OAV) allows one to estimate the contribution of a specific compound to the aroma of wine (Peinado *et al.*, 2004).

The aroma is subdivided into varietal or primary aroma, fermentative or secondary aroma and post- fermentative or terciary. In young white wines, like ones considered in the present study, the total aroma has a strong varietal and fermentative component, and a lesser extent of post-fermentative.

It has been widely accepted that primary wine aroma come from those compounds located in grape, including both found in grape skill and pulp (Ribereau-Gayón *et al.*, 2000; Flanzy, 2000). These compounds obtained through grape metabolism, vary as a function not only of the variety but also of certain cultural and climate-related factors, including geographical, cultural and viticultural factors and wine-making techniques (Bureau *et al.*, 2000). These compounds have a great importance because they play a key role in the differenciation of wines according to distinct grape varieties used for winemaking.

Aromatic compounds are present as free forms, which may contributed directly to odour and, and in much larger concentrations as non-volatile forms called aroma precursors, among them the sugar-bound conjugates being the most abundant (Gunata, *et al.*, 1985). In order to release glycosylated compounds and enrich wine aromatic profile, it can be carried out a chemical hydrolysis during ageing or an enzymatic hydrolysis along winemaking using commercial enzyme preparations with β-glucosidase activity (Carballeira *et al.*, 2001). Any microorganims with this enzymatic activity could also release bound aromatic compounds.

Among free volatiles, monoterpenes, due to their high concentration and lower aroma threshold, are considered the main components responsible for the primary aroma of these white young wines (Zamúz *et al.*, 2006). These are mainly derived from grape skins and are synthesized during ripening. Apart from monoterpenes, pyrazines and some alcohols are also presented in grapes. These compounds, which are secondary products of the metabolism of the plant, are distributed between the pulp and grape skin, wherein their concentration is higher (Fernández *et al.*, 1999).

Carotenoids are an important group of aroma precursors. These organic pigments are synthetized naturally in grape and are known as precursors of norisoprenoids, responsible for the typical aroma of same grape varieties. C13-norisoprenoids are in grapes mainly as glycosilated compounds (Flanzy, 2000). All these molecules have an important sensorial impact on wine aroma as they have very low olfactory perception thresholds providing floral and fruity aromas (Peynaud *et al.*, 1996).

Regarding to fermentative or secondary aromas, it is well known that most of them are produced along alcoholic fermentation and their concentration mainly depends on predominanting yeasts during fermentation and the conditions under which it is performed (Steger *et al.*, 2000; Egli *et al.*, 1998). Yeasts contribute to the formation of aromatic

compounds, because they have the capacity to synthesise novo yeast-derived volatile compounds during fermentation (Swiegers, *et al.*, 2006; Ugliano *et al.*, 2006; Wondra *et al.*, 2001). Derived from the metabolism of carbohydrates, yeasts produce organic acids, higher alcohols, esters and to a lesser extent, aldehydes (Rapp *et al.*, 1991). The consequences of the yeast activity in final aroma, can be determined by instrumental or sensory analysis.

As we commented before, the spontaneous alcoholic fermentation of grape must is a complex process carried out by many different indigenous yeasts. The diversity and composition of yeast populations can influence to a large extent the chemical composition of wines, and therefore it's final aroma and flavour profile (Swiegers et al., 2005; Lurton et al., 1995). These authoctonous mircroorganisms can also be inoculated as starter cultures previosly selected (Ciani, 1997; Boulton *et al.*, 1996; Fleet *et al.*,1993). Precisely, a first study about indigenous mircrorganisms in Galician grape musts was carried out in the Microbiology Department of the Industrial Fermentation Institute of CSIC by members of the so-called College of Oenological Microbiology in Madrid (Carrascosa, 2010). The aim of this study was the isolation, identification and characterization of yeasts, in order to select best suited. Results obtained confirm that spontaneous fermentations sometimes become stuck or sluggish. Due to the lack of reproducibility and predictability of spontaneous fermentations and in order to have a better microbiological and fermentation control, the inoculation of selected cultures of *S. cerevisiae* was proposed as a possible solution. Even though, commercial starter cultures of *S. cerevisiae* are widely available for fermentation of the must in wineries, several studies support the hypothesis that active dried yeasts reduce the variability of strains that appear in spontaneous fermentation (Beltran *et al.*, 2002; Frezier *et al.*, 1991) and, possibly, the complexity of the resulting wine. For this reason, wineries looking to enhance the sensorial quality of wines prefer the use of selected local *S.cerevisiae* strains for the fermentation of their musts that could affect positively the final quality of wines

(Romano *et al.*, 2003). In fact, *S. cerevisiae* starter cultures used in some wineries have excellent results (Estévez et al., 2004; Nurgel *et al.*, 2003) and in general, the quality of these wines is better quality than those obtained by spontaneous fermentation (Regodón *et al.*, 1997, Clemente-Jimenez *et al.*, 2004), with similar organoleptic characteristics steady through years (Bauer *et al.*, 2000). Therefore, the use of local selected yeast could be a sucessful strategy to modulate wine aromatic profiles and improve sensorial quality of wines.

The effect of *S. cerevisiae* strain and the influence of growing are in the aromatic profile of Alabriño wines has been studied before (Vilanova *et al.*, 2006; Vilanova *et al.*, 2005). Nevertheless, at the time of this study, the influence of a native *S. cerevisie* strains on terpene and norisoprenoid concentrations was not adressed. In the chapter 1 of the current thesis, results obtained from this first study in Albariño wines are presented.

Phenolic compounds or polyphenols are natural constituents of grapes and wines. Under the name of wine polyphenols, numerous compounds of different chemical structures are mainly grouped into non flavonoids, which are hydroxybenzoic acids, hydroxycinnamic acids, stilbenes and phenolic alcohols, and flavonoids, flavonols, flavan-3-ols, anthocyanins and other flavonoids. They contribute to the organoleptic characteristics of wine, such as colour, aroma, astringency and bitterness, and have been associated with positive nutritional and pharmaceutical properties.

White wines contain significantly lower amounts of total polyphenol than red wines, mainly hydroxicinamic and benzoic acids, flavan-3-ols and flavanols, which represent an average of 10.38 mg/100 ml, depending on the variety of grape and oenological factors (Neveu *et al.*, 2010). Phenolic compounds, although to a lesser extent, also contribute to the fermentative or secondary aroma of wines. Their presence in the must not significantly affect the growth of yeasts, although it has been studied that anthocyanins of red musts may

enhance it's development and proantocyanidins of white wines are able to obstruct or extent the replication of *S. cerevisiae* (Howitz *et al.*, 2003). Aditionally, some studies have confirmed the influence of yeast strains in the phenolic composition of wines (Sacchi *et al.*, 2005; Caridi *et al.*, 2004). Phenolic composition can also affect the aroma of the wine, and it has been demostrated, for example, that polyphenols can inhibit the decrease of terpenes and other volatile compounds (Lambropoulos *et al.*, 2007; Roussis *et al.*, 2007). Likewise, the presence of volatile phenols in white wines, 4-vinylphenol and 4-vinylguaiacol obtained from the decarboxylation of p-coumaric and ferulic acid, respectively, via *S. cerevisiae* cinnamate decarboxylase, can also influence negatively the aroma (Chatonnet *et al.*, 1997*).*

The effect of indigenous *S. cerevisie* strains in the phenolic composition of Albariño white wines is explained in the chapter 1 of the current thesis. This is the first time that this effect has been studied in Albariño white wines from Rias Baixas PDO.

3.2 Mannoproteins.

S. cerevisiae is recovered with an organelle called cell wall, which is essential for it's survival (Cabib *et al.*, 2001), and accounts for about one third of the cell wall dry weight (Lesage *et al.*, 2006, Klis *et al.*, 2006). Located on the cell wall external layer, there are mannoproteins that represent 35-40% of the dried weight of cell wall (Quirós *et al.*, 2010).

These polysaccharides are glycoproteins composed with 30% of protein and 90% of polysaccharide of which 98% is mannose and 2% is glucose (Waters *et al.*, 1994).

The release of mannoproteins into wine can occur in two different processes: during alcoholic fermentation in the yeast growing phase until a concentration between 100-150 mg/L with widely varying sizes ranging from 5000 Daltons (Da) to 800,000 (Da) (Doco *et al.*, 1996) and those release after yeast autolysis of dead yeast by the action of endo and exo β-

1,3-glucanase enzymes on the yeast cell walls, as occur, for example, in the ageing on less. These last mannoproteins are similar to those released during fermentation but they use to have less protein content (Saulnier *et al.*, 1991).

Mannoproteins are known for several important oenological properties in wines. They contribute to the chemical stabilization of wine by preventing the crystallisation of tartrate salts and protein haziness (Moine-Ledoux *et al.*, 1999; Gonzalez-Ramos *et al.*, 2009). Besides, mannoproteins can interact with some wine aromas, improving sensorial characteristics of wines. According to the study of Lubbers *et al.*, 1994, there is an interaction of mannoproteins with a high protein content with volatile compounds like β-ionone, ethyl hexanoate and hexanol. A positive effect of mannoproteins in wine aroma was also described by Chalier *et al.*, 2007. Furthermore, these colloids can interact with wine phenolic compounds improving sensorial characteristics, namely the impact in colour stability (Bellachioma, 2002), the reduction of red wine astringency (Escot *et al.*, 2003) and the increase of the body, sweetness, roundness and mouthfeel of the final wine (Guadalupe *et al.*, 2007; Vidal *et al.*, 2003). Mannoproteins can also contribute to the improvement of the foam quality of sparkling wines (Nuñez *et al.*, 2006), and in the reduction of the concentration of some undesirable compounds such as ochratoxin A (Núñez *et al.,* 2008). Finally, there are studies that confirm the use of mannoproteins to stimulate the growth of lactic acid bacteria in wine environment and thus the development of malolactic fermentation (Rosi *et al.*, 1999; Guilloux-Benattier *et al.*, 1995).

The content of mannoproteins in Albariño wines and their correlation with the sensory quality of wines had not been addressed previously, so considering their positive oenological properties and given that approximately the 32% of the total polysaccharides in white wine are mannoproteins (Gonçalves *et al.*, 2002), an individual study in Albariño white wines was conducted. Results obtained from this study are presented in the chapter 2 of the current

report.

The ageing on lees is a widely used oenological practice for quality wines, consisting in letting the wine to stay with resting yeast cell. Along this period of dead yeast in contact with wines, and in the stirring of these lees known as of "battonnage" practice, there is a sucession of processes due to the action of microorganisms, which contribute positively to the sensory characteristics of the wine and also to it's enrichment in mannoprotein (Muñoz *et al.*, 2011). In particular, the wine acquires aromatic complexity as a result of important modifications deriving from reactions such as esterification, hydrolysis, and redox reactions (Camara *et al.*, 2006).

Once again, the positive effect on ageing on lees in the organoleptic characteristics of some white wines and it's relationship with a distinctive and complex character, gave us the idea to carry out a study to analyse the effect of short ageing on lees in Albariño wines, which results were presented in the chapter 3 of this thesis.

The important effect of *S.cerevisiae* in alcoholic fermentation and in final quality of wines, has produced a big interest in wine microbiology. In the last 20 years, many efforts have been made to modify the genome of wine strains by classical techniques: mutagenesis and selection, hybridization, cytoduction, and protoplast fusion.

The emergence of recombinat DNA and all scientific knowledge about the biochemistry, genetics, and molecular biology of *S. cerevisiae* has allowed the use of genetic engineering as a more specific and safely strategy to improve genetically yeasts involved in winemaking. All these strategies, must guarantee the fermentative capacity of modified yeasts (Gimeno *et al.*, 2001).

Although there is an important number of aspects that could be improved on

S.cerevisie through genetic enginering (Pretorius, 2003), those that contribute to the improvement of it's quality are most interesting for the consumer. Regarding to nutritional quality, there is a possibility to increase the concentration of nutritional compounds and to dicrease the antinutritional or toxic ones (González *et al*., 2011). For example, transgenic yeast strains capable to increase the concentration of resveratrol in wines have been successfully developed (González-Candelas *et al*., 2000; Becker *et al*., 2003). Concerning the control of toxic compounds, a transgenic yeast with the capacity to reduce the concentration of ethyl-methyl-carbamate in wines has been developed (Coulon *et al*., 2006) .

In the case of sensorial quality, a *S. cerevisiae* wine strain over-producing an endogenous exoglucanase has been successful constructed to research the possible role of this enzyme in increasing wine aroma through the release of a glycosidic precursor, instigating a substantial improvement in it's primary aroma components (Pérez-González *et al*., 1993).

Moreover, Gonzalez-Ramos *et al*., 2008, under the supervision of Dr. Ramón González, have obtained a transgenic over-producing mannoproteins strain that could increase their content in wines and protect the wine agaisnt protein haze. Precisely, the use of this transgenic yeast in the winemaking of Albariño wine and it's influence on sensory quality was an unpublished subject that we decided to address. The results of this study are presented in chapter 4 of the present thesis.

4. Lactic bacteria and sensorial quality of wines.

At harvest, grape berries carry lactic acid bacteria, besides yeasts, acetic acid bacteria and molds. Yeasts and lactic acid bacteria are the main microorganisms involved in

winemaking. After alcoholic fermentation, when all reducing sugars are fermented to ethanol, yeast levels decline and lactic acid bacteria growth occurs. Malolactic fermentation (MLF) then follows the alcoholic fermentation (Lonvaud-Funel., 1999). This process, involves the bioconversion of the malic acid present in wine into lactic acid and carbon dioxide, which is done by wine lactic acid bacteria (LAB).

Besides deacidifying the wine, this fermentation improves the biological stability, decreasing the possibility of refermentation once the wine is bottled, and enhance it's organoleptic characteristic, as lactic acid is less bitter than the malic acid, and less astringent (Muñoz *et al.*, 2011). Moreover, during MLF the bacteria also can affect the final aroma balance and flavour by various mechanisms including the production of volatile secondary metabolites and the modification of grape and derived-yeast metabolites (Davis *et al.*, 1985; Henick-Kling, 1995).

While it is widely recommended in red wines, the malolactic fermentation it is just carry out only in certain style of white wines, because normally decreases the typical fresh and herbal character of these wines. Also, the lower pH of white wines, is a stress factor that when is combined with other oenological parameters, can influence the survival of LAB and consequently MLF (Lonvaud-Funel, 1995). Therefore it is not surprising the absence of data in young white wines such as ones studied in this report: Caiño and Albariño wines.

This secondary fermentation is conducted by species of the genera *Pediococcus*, *Lactobacillus*, *Leuconostoc* and *Oenococcus oeni*, previously *Leuconostoc oenos* (Bartowsky, 2005). However, in most cases, it is carried out by *Oenococcus oeni* (*O.oeni*) species since they are the best adapted to the low pH and high ethanol concentration conditions of wine (Hernandez-Orte, *et al.*, 2009). Along with *O. oeni*, *Pediococcus damnosus (P. damnosus)* y *Leuconostoc mesenteroides (L. mesenteroides)* are considered

principal responsible of MLF in wines (Lonvaud-Funel *et al*, 1999). While *O. oeni* are mainly presented in wines, *Lactobacillus plantarum* (*L. plantarum*), *Lactobacillus casei* (*L. casei*) *Lactobacillus hilgardii (L.hildargii)*, *L. mesenteroides* y *P. damnosus* are in must (Muñoz *et al*., 2011).

O. oeni disappears quickly after malolactic fermentation, remaining in wine *Pediococcus* and *Lactobacillus* strains. In case of a low acid wine that is not sulfited, some strains can degradate other wine compounds such as citric acid, tartaric acid, etc., affecting negatively the wine. Moreover, it has also been proven that *P. damnosus* strains produce an active bacteriocin against *O. oeni* (Green *et al*., 1997).

In winemaking, although there are many commercial starters, it is still common to conduct a spontaneous malolactic fermentation, enhanced by an appropriate incubation temperature in the cellar. Nevertheless, as it happened with yeast, there is a increasing trend towards the use of autochthonous starter culture, well adapted to the conditions of a specific wine-producing area, to ensure the reproducibility of wines (González *et al*., 2011).

LAB can have a beneficial or detrimental effet in the quality of wine, depending on the specie, even on the strain, and on the stage at which they appear in the vinification process (Lonvaud-Funel, 1999). Biogenic amines are compounds harmful to human health and negatively affect the safety of the wine (Lonvaud-Funel., 2001; Arena *et al*., 2001). Different results have been reported for biogenic amine production by lactic bacteria strains, mainly from the genera *Lactobacillus and Pediococcus*, therefore it is most desirable to be able to avoid the formation of these amines during MLF (Costantini *et al*., 2006; Landete *et al*., 2007a). Likewise, among the number of different ways in which *Pedicococcus* can alter a wine, one of the most problematic of these alterations is the abilty to synthetize extracelullar polysaccharides that cause an increase in the viscosity of the wine, creating problems during

filtration and giving the wine a “ropy” apperance (Fugelsang, 1997; Manca de Nadra *et al.*, 1995). This can cause severe losses to winemaker as the wine quality is often irrecoverable (Walling *et al.*, 2005). Although these negative effects, previous studies (Edwards *et al.*, 1992,1994) have confirmed that the growth of *pediococci* could be interesting in wines as it may add desirable flavors and aromas under certain circumstances without any spoilage effect.

Taking into consideration all these effects, it is convenient the selection of potential starter cultures from native strains for malolactic fermentation, to verify the lack of possible undesirable properties (González *et al.*, 2011).

Besides the conversion of malic acid in lactic acid, which is the principal reaction in terms on quantity, malolactic fermentation involves many other reactions that affect the final organoleptic quality of wines.

4.1 Aroma in malolactic fermentation.

According to various studies, it is well known that MLF affects wine aroma and flavour, adding complexity depending on the strain used and the type of wine (Delaquis *et al.*, 2000; Sauvageot *et al.*, 1997; Henick-Kling, 1993; Rodríguez *et al.*, 1990; McDaniel *et al.*, 1987).

The metabolic activity, as well as the kinetics of MLF, will influence the sensory profile of the wine related to vinification techniques, the physical and chemical composition of the wine (Krieger-Weber, 2009).

Their influence in aroma can be due to the effect of LAB during malolactic fermentation in the reduction of varietal aromas by hydrolysis or degradation of aromatic compounds of grapes, as well as secondary produced during alcoholic fermentation, or by

simply bubbling that *O. oeni* unleashes due to it's heterofermentative metabolism. It has been demostrated that malolactic fermentation increase fruity and butter notes and decrease green and herbal aromas in wines (Henick-Kling,1993).

A higher concentration of fruity aromas is caused by the presence of esters in wines. Ester hydrolysis and synthesis can be catalysed by esterases. Some authors have reported the presence of esterase activity in *Oenococcus*, *Pediococcus* and *Lactobacillus* from wine (Matthews *et al.*, 2004, 2006, 2007, Davis *et al.*, 1988) that could be related to a higher presence of esters as ethyl lactate, ethyl acetate, ethyl hexanoate and ethyl octanoate, which are formed during MLF (Delaquis *et al.*, 2000; De Revel *et al.*, 1999, Ebeler, 2001).

Acetaldehyde is one of the major carbonyls produced during alcoholic fermentation and of great importance in wine color, aroma and microbiological stability (Lui *et al.*, 2002). The excess of acetaldehyde, hexanal, cis-hexen-3-al, and trans-hexen-2-al lead the apperance of unpleasant odors. Their higher content is mainly controlled by the adiction of SO_2, but recently some studies have demostrated the degration of acetaldehyde by lactic into ethanol and/or acetic acid (Osborne *et al.*, 2000), contributing to the reduction of herbaceous aroma of wines.

Diacetyl, which imparts a buttery aroma and flavour in wines, is a major flavour metabolite produced by lactic acid bacteria (LAB). The biosynthesis of diacetyl depends on the metabolism of citric acid where it is an intermediate metabolite that can be further reduced to acetoin and the alcohol, 2,3-butanediol (Bartowsky *et al.*, 2004). At low concentrations (and combinated with other wine aroma compounds), this compound will impart yeasty, nutty, toasty aromas (Eteviant *et al.*, 1991). When it is presented at a high concentration (exceeding 5–7 mg/l), diacetyl is considered by many to be undesirable in wine, adding a characteristic buttery aroma that is associated with a lactic character.

(Bartowsky *et al.*, 2000; Nielsen *et al.*, 1999). The reaction between diacetyl and sulfur dioxide, which is reversible and exothermic, can be used to suppress the buttery aroma in wine. Factors such as the temperature of the wine, and time will influence this interaction and thus the sensory perception of diacetyl (Nielsen *et al.*, 1999).

On the other hand, mono-, di(-) and triacylglycerols that we found in wines derived from grapes or released during yeast autolysis (Pueyo *et al.*, 2000), can be a source of volatile fatty acid with low perception threshold, which are synthetyzed by lipase enzymes from LAB presented in wine (Davis *et al.*, 1988).

Moreover, some studies have determined the ability of several lactic bacteria to change the volatile fraction of wine by releasing aroma compounds (such as volatile phenols, terpenes, lactones, norisoprenoids and vanillins) from grape flavour precursors. This is a direct effect of the glycosidase activity that have some of these LAB (D'Incecco *et al.*, 2004; Grimaldi *et al.*, 2000; McMahon *et al.*, 1999).

Regarding MLF, it has been empirically known for years that the phenolic content of grapes and wines can affect the rate and extent of this fermentation (Campos *et al.*, 2009). Some studies have demostrated that some phenolic acids can have a positive or negative effect in the growth of lactic acid bacteria, depending on the nature and concentration of the compound and on the strain (Reguant *et al.*, 2000; Salih *et al.*, 2000; Rozès *et al.*, 1998). Furthermore, these phenolic acids are also recognized for delaying the metabolism of sugars and citric acid by wine lactic bacteria (Rozès *et al.*, 2003). It has been reported that strains with cinnamoyl esterase activity can hydrolase caftaric and coutaric acids during malolactic fermentation increasing the concentration of the corresponding free hydroxycinnamic acids (Hernández *et al.*, 2007). Other authors have demostrated that some lactic bacteria are able to produce volatile phenols (vinylphenols and ethylphenols) from the metabolism of

hydroxycinnamic acids (Rodríguez *et al.*, 2008b; Landete *et al.*, 2007b; Cavin *et al.*, 1993). Further works have showed the capacity of some strains of *Lactobacillus plantarum* to degrate hydroxybenzoic acids to produce some compounds such as catechol that benefits the growth and metabolism of this bacterium (Alberto *et al.,* 2004; Rodriguez *et al.*, 2008a).

There is evidence for the presence of proteins in wines that can be hdrolyzed by bacteria proteases and peptidases to produce peptides and amino acids thay could influence the flavour and stability of wine, although these effects and activities are not significant relevant in wines fermented by LAB (Leitao *et al.*, 2000; Manca de Nadra *et al.*, 1997, 1999).

Other aromas associatted with MLF are floral, toasty, vanilla, sweet, wood, smoky, bitter and honey notes (Sauvageot *et al.*, 1997; Henick-Kling, 1993). Additional analyses are required to connect the presence of these descriptors with a chemical modification of a specific compound along the MLF. As in the preceding revision of yeasts, further studies of these secondary activities in the MLF, will allow the isolation and selection of adequate LAB strains that will permit the winemaker to obtain wines with certain sensory characteristics.

4.2 Colour in malolactic fermentation.

Colour is one of the most important sensory characteristics of wines, which influences the consumers' overall acceptability. Numerous factors can affect wine colour, such as the grape composition, the vinification method and the storage conditions (Gil Muñoz *et al.,* 1997). As we commented before, phenolic compounds are principal responsible for some of the major organoleptic properties of wines as the colour.

In white wines, which contain significantly lower amounts of total polyphenols compared with red wines, phenolic compounds contribute to the colour stability because they can act as oxidation substrates in white wines (Karagiannis *et al.*, 2000, Soleas *et al.*, 1997). Therefore, the colour in white wines changes due to their browning, lossing their bright pale-

yellow colour, which might occur when the total phenolic content is too high (Benítez *et al.*, 2002).

The effect of MLF in wine color normally entails a decrease in color intensity. In the case of white wines it could produce a descent in bright- yellow notes while in red wines in blue ones, which is mainly due to the possible adsorption of anthocyanins, fundamentally methoxylated, by bacterial cell walls that also help the rise in pH because the t transformation of malic acid into lactic acid, and the decrease of levels of free sulfur dioxide (Suárez-Lepe *et al.*, 2003). On the other hand, through the release of glycosylated precursors, LAB might influence wine color due to it's β-glycosylated capacity (Grimaldi *et al.*, 2000).

4.3 Body in malolactic fermentation.

Besides the aroma, it is thought that the malolactic fermentation increases the body, smoothness and roundness of wine due to the production of polyalcohols and polysaccharides by lactic acid bacteria.

Wine LAB can synthesize polyalcohols such as glycerol or erythritol (Liu *et al.*, 1995; Firme *et al.*, 1994). The production of polyalcohols can influence the organoleptic characteristics of wine such as the body, creaminess and roundness, and some technological aspects like filtration. Some other LAB have the capacity to degrate these polyalcohols into precursors such as acrolein. Acrolein itself is not a bitter compound, but it thought to interact with as yet undetermined phenolic compounds in wine to contribute bitterness at concentrations as low as 10ppm (Bauer *et al.*, 2010). Furthermore, Vaquero *et al.*, 2004 have described a tanase activity in *L.plantarum* that affect both the color and turbity of final wines.

In chapter 5 are presented results obtained in the study of the effect of malolactic fermentation in both white Caiño and Albariño wines. Since this topic has not been previously addressed by any author, an isolation of beneficial authoctonous LAB was carried out in order to verify their effect in the final sensorial quality of wines.

CHAPTER 1

Influence of locally-selected yeast on the chemical and sensorial properties os Albariño white wines.

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CAPÍTULO 1

INFLUENCIA DE UNA CEPA AUTÓCTONA DE *SACCHAROMYCES CEREVISIAE* SOBRE LA COMPOSICIÓN QUÍMICA Y LA CALIDAD SENSORIAL DE VINOS BLANCOS ALBARIÑO

OBJETIVO

El objetivo del presente capítulo fue el de obtener una cepa de levadura idónea para llevar a cabo la vinificación controlada de mostos de *Vitis vinifera* cv. Albariño de la D.O. Rías Baixas. En el momento de realizar este trabajo, no se había hecho estudio similar alguno sobre tal tipo de vinos, y la mayor parte de las bodegas de la zona llevaban a cabo la fermentación espontánea de sus mostos o añadían inóculos de levaduras comerciales. La selección final de la cepa más idónea, que es la parte que se recoge en este capítulo, se realizó a partir de tres cepas de la especie *Saccharomyces cerevisiae* (*S.cerevisiae*) que en dos vendimias consecutivas realizadas con anterioridad a estos ensayos, consiguieron predominar sobre el resto de la microbiota salvaje, en vinificaciones espontáneas correctas que dieron como resultado vinos de Albariño típicos y sin defectos organolépticos.

PLAN DE TRABAJO

Para la realización del objetivo propuesto se llevaron a cabo las siguientes tareas:

1. Comprobación *in vitro* de la capacidad de imposición de las cepas de *S. cerevisiae*, mediante la realización de microvinificaciones (1L), utilizando la técnica de tipado molecular del ADN mitocondrial.

2. Estudio *in vitro* de la dinámica fermentativa de las vinificaciones realizadas con las cepas de *S. cerevisiae* seleccionadas, mediante la realización de

microvinificaciones (1L), determinando mediante análisis instrumental el grado alcohólico, la acidez total, la acidez volátil y el pH.

3. Comprobación en bodega de la capacidad de imposición de las cepas de *S. cerevisiae* pertenecientes a la colección estudiada, mediante la realización de vinificaciones a escala piloto (30 L), utilizando la técnica de tipado molecular del ADN mitocondrial.

4. Estudio en bodega de la dinámica fermentativa de las vinificaciones realizadas con las cepas de *S. cerevisiae* estudiadas, mediante la realización de vinificaciones a escala piloto (30 L), determinando mediante análisis instrumental el consumo de azúcar.

5. Estudio de los vinos obtenidos por vinificación a escala piloto en bodega, por análisis instrumental mediante la identificación y cuantificación de los compuestos volátiles y la identificación y cuantificación de los parámetros de color y de los compuestos fenólicos.

6. Análisis sensorial de los vinos obtenidos y su relación con la composición química.

RESUMEN

En este trabajo se estudiaron los cambios en la composición química (compuestos del aroma y compuestos fenólicos) y en las propiedades sensoriales producidos por la fermentación del mosto Albariño con levaduras seleccionadas del entorno de producción de estos vinos (cepas de *S. cerevisiae* 1, 2, 3). Como control se utilizó un vino obtenido por fermentación espontánea del mosto Albariño, que era la forma más habitual de la bodega colaboradora de obtener sus vinos. Se pudo comprobar que el porcentaje de imposición de las cepas inoculadas en todos los tanques de fermentación estuvo entre el 90% y el 100%, mientras que el mosto no inoculado presentó una población heterogénea de levaduras que

incluyó también a las cepas 1 y 3 con un porcentaje de imposición del 43% y el 20%, respectivamente. Los mostos inoculados fermentaron con mayor rapidez (25 días) que el correspondiente a la fermentación espontánea (35 días). Los resultados obtenidos indicaron que la cepa de levadura influye significativamente tanto en la composición de los compuestos del aroma como de la fracción fenólica. Con respecto a los compuestos del aroma, se observó que los vinos elaborados con la cepa *S. cerevisiae* 1 presentaron una concentración significativamente mayor de terpenos y norisoprenoides, a pesar de haber sido elaborados con el mismo mosto. Por ejemplo, el geraniol y el linalool se detectaron a concentraciones superiores a su umbral de percepción (130 y 50 mg/L respectivamente). También los norisoprenoides α-ionona y β-damascenona presentaron concentraciones superiores en estos vinos. Estos compuestos están muy relacionados con el carácter distintivo frutal y herbáceo de los vinos blancos Albariño. En cuanto a su composición fenólica, los vinos fermentados con la cepa *S. cerevisiae* 1 presentaron una concentración de catequinas, flavan-3-oles, procianidinas y ácido hidroxicinámico y derivados significativamente inferior al resto de los vinos. Muchos de estos compuestos están relacionados con características sensoriales desfavorables para este tipo de vinos, como son una mayor astringencia y amargor. Por otro lado, dichos vinos presentaron un potencial de pardeamiento significativamente inferior al resto, lo que también está relacionado con la concentración de flavan-3-oles y derivados del ácido hidroxicinámico, que se asocian a los fenómenos de pardeamiento en vinos blancos. Los vinos fermentados con la cepa *S. cerevisiae* 1 presentaron el mejor perfil sensorial. Teniendo todos los vinos el mismo aspecto visual, los vinos fermentados con la cepa *S. cerevisiae* 1 tuvieron mejores atributos de aroma y sabor, lo que guarda una relación directa con lo dicho más arriba. Los vinos peor calificados en el análisis sensorial fueron los realizados mediante fermentación espontánea. Por todo ello la cepa seleccionada como la más idónea para la elaboración de vinos típicos de *Vitis vinifera* var. Albariño fue *S. cerevisiae* 1.

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Influence of locally-selected yeast on the chemical and sensorial properties of Albariño white wines

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ABSTRACT

The use of selected yeast strains with improved or novel properties may promote wines with special and original quality attributes. In this paper, changes in the chemical composition (aroma compounds and polyphenols) and sensorial properties of Albariño white wines elaborated with the same must and selected yeast (named as 1, 2 and 3) have been studied in comparison with wines subjected to non-inoculated fermentation (control wine). The results indicated that yeast strain can significantly influence the aroma and polyphenol composition of the wines. Wines elaborated with strain 1 had a higher concentration of terpenes and norisoprenoids, which are compounds closely associated with the fruity and fresh character of Albariño white wines. These same wines had a lower concentration of flavan-3-ols, closely associated with the astringency and bitterness of the wine and the lowest browning potential. The formal sensory analysis conducted by 8 trained judges showed that wines elaborated with strain 1 were preferred by the tasting panel. Therefore, the selection of yeast strains could offer the possibility to modulate sensorial attributes related with the aroma and phenol composition in Albariño white wines.

1. Introduction

Vitis vinifera cv. Albariño is an aromatic white variety of grape grown in Galicia, Spain. Albariño grapes are used to elaborate high-quality white wines of characteristic fruity and floral aromas. In young white wines made from Albariño grapes, these floral notes have mainly been attributed to free monoterpenes and norisoprenoids (Carballeira, Cortes, Gil, & Fernandez, 2001). These compounds are predominantly derived from the grape, synthesized during maturation, and qualitatively and quantitatively influenced by several variables which could strongly affect the aromatic composition of wine. The incidence of these variables, such as the climate, soil, and viticultural practices has been intensively investigated (Bureau, Razungles, & Baumes, 2000; Dieguez, Lois, Gomez, & de la Peña, 2003). However, the influence of the yeast strain used to ferment the must on the terpene and norisoprenoid composition and the sensorial character of these white wines have not been clearly established. Moreover, today many wineries which use Albariño grapes to elaborate white wines are still carrying out "spontaneous" fermentation of the must instead of inoculating it with selected yeasts.

Different strains of *Saccharomyces cerevisiae* can produce significantly different flavor profiles when fermenting the same must. This is a consequence of the differential ability of wine yeast strains to release varietal volatile compounds from grape precursors, and/or a different capacity to synthesize yeast-derived volatile compounds (Papathanasiou, Selvagini, Servili, Vaughan-Martini, & Roussis, 2006; Ugliano, Bartowsky, McCarthy, Moio, & Henschke, 2006; Vilanova & Sieiro, 2006). Regarding the Albariño wines, there are limited and contradictory data on the feasibility of inoculating the must with selected yeast. While some studies have shown that wines obtained by spontaneous fermentation are more aromatic than those obtained by inoculating a selected yeast strain (Vilanova & Sieiro, 2006), others have reported that the use of locally-selected yeast strains can positively affect the final quality of the wine (Vilanova & Massneuf-Pomarede, 2005).

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On the other hand, it has also been shown that the yeast strain used to ferment the must can also influence the phenolic composition of the wine (Caridi, Cufari, Lovino, Palumbo, & Tedesco, 2004; Sacchi, Bisson, & Adams, 2005). Phenolic compounds are partly responsible for the color, astringency and bitterness of wine, as well as for numerous physiological properties associated with wine consumption (Flanzy, 2003). In white wines, phenolic compounds mainly include hydroxycinnamic and benzoic acids, flavan-3-ols and flavonols and account for 10.38 mg/100 mL (as an average), depending on grape variety and cultivar, and enological factors (Neveu et al., 2010). Phenolic composition has been related to wine aroma as polyphenols have been shown to inhibit a reduction in terpenes and other volatile compounds (Lambropoulos & Roussis, 2007; Roussis, Lambropoulos, & Tzimas, 2007), helping to maintain the typical aroma of these wines. The purpose of the present study was, therefore, to determine the influence of locally-selected yeast on the sensorial character of Albariño white wines, determining key aroma compounds in these wines and also analyzing the influence of the yeast on their phenolic composition.

2. Material and methods

2.1. Must, yeasts and fermentation conditions

The must used in this study was from *V. vinifera* cv. Albariño grapes and was supplied by the winery Terras Gauda, Galicia, Spain.

To carry out the fermentation, three different yeast strains (1, 2 and 3) were used. These strains were isolated from different vinification processes in the wine cellar from two previous vintages produced without yeast inoculation. In all cases, they were the predominant yeast at the end of the fermentation. Microvinifications (1 L) were previously done in the laboratory to check the fermentative capacity of selected yeast at 18 °C (Quirós, Morales, Pérez-Través, Barcenilla, & González, 2011) and the following chemical characteristics of the wines (Alcoholic grade, total acidity, volatile acidity, and pH). All the parameters were determined by the European Commission methods (European Community, 1990). For the experiments developed in the cellar, the must was inoculated with yeast and fermented in 30 L stainless steel tanks. Temperature was set to 18 °C. A control wine (C) was prepared by fermenting the must with its own yeast. In all cases, experiments were carried out in triplicate. Predominance of the selected yeast in the fermentation tanks was verified by studying the mitochondrial DNA profile in different stages of the fermentation (initial phase: 3 days, middle fermentation: 15 days, and at the end of fermentation: 25 days), following the method described by Querol, Barrio, & Ramon, (1992). Fermentation was followed by the sugar consumption in each tank and the reducing sugar (Flanzy, 2003) during fermentation was determined until 40 days. When fermentation was complete in each case, 250 mL samples were taken from each tank. Samples were cleared by centrifugation at 10000 rpm, for 15 min, and were used for the determination of volatile and phenolic compounds.

2.2. Volatile compounds

Analysis of the major volatile compounds (higher alcohols) was performed by direct injection in a Hewlett–Packard (Palo Alto, CA) 5890 series II gas chromatograph equipped with flame ionization detection (FID) and a split/splitless injector. Separations were carried out on a Chrompack CP-WAX 57 CB fused silica capillary column (50 m × 0.25 mm i.d.) coated with a 0.25 μm thick polyethylene glycol stationary phase (Varian, Houten, The Netherlands) and the injector and detector temperatures were 220 °C. The temperature program was as follows: initial temperature 40 °C (10 min hold) and ramps of 7 °C/min to 150 °C and 30 °C/min to 210 °C. The carrier gas was helium (15 psi). A total of 50 μL of 3-pentanol (6 mg/mL 10% ethanol) was added as internal standard to 10 mL of wine, and 2 μL of wine with the internal standard was injected in the split mode. A ChemStation data system (HP 3365 series II, v. A.03.21) was used for data acquisition and processing. The compounds determined by this method were methanol, hexanol, 2-phenylethanol, 1-propanol, 2-methyl-1-propanol, and 2- and 3-methyl-1-butanol.

Minor volatile compounds (esters and acetates) were analyzed by gas chromatography of the headspace extract obtained with a 100 μm poly-dimethylsiloxane coated fused silica fiber (Supelco, Bellefonte, PA), in the conditions described by Pozo-Bayon, Pueyo, Martin-Alvarez, and Polo (2001), using methyl nonanoate as internal standard. The compounds determined by this method were ethyl butyrate, ethyl hexanoate, hexyl acetate, butyl acetate, isobutyl acetate, isoamyl acetate, ethyl octanoate, ethyl decanoate, hexanoic acid and octanoic acid. The temperature of both injector and detector was 250 °C. The temperature program was as follows: initial temperature 70 °C (5 min hold) and ramps of 5 °C/min to 200 °C and 3 °C/min to 215 °C. The carrier gas was helium (15 psi).

Free terpenes and norisoprenoids were fractionated by selective retention on SepPak Vac C-18, according to the procedure described by Vilanova and Sieiro (2006). The free fraction was eluted with pentane dichloromethane (2:1, 10 mL) and the eluate was dried over anhydrous sodium sulfate and concentrated to 0.5 mL, by evaporation with a stream of nitrogen, before GC analysis. Conditions used for chromatographic analysis were: injector temperature (250 °C), detector temperature (260 °C), injection type (Splitless, 30 s) and injection size (1 μL). The temperature program was as follows: initial temperature 70 °C (5 min hold) and ramps of 2 °C/min to 120 °C and 3 °C/min to 215 °C during 25 min. The carrier gas was helium (14.5 psi).The compounds determined by this methodology were geraniol, linalool, nerol, α-ionone and β-damascenone, using 3-octanol as internal standard.

In all cases, peak identities were assigned by comparing the relative retention times of the internal standard, with those of the standards of analytical quality, of over 99% purity, from Sigma–Aldrich (St. Louis, MO). For quantification purposes, the relative area was obtained as the chromatographic peak area of each aroma compound divided by the area of the internal standard. Calibration curves in synthetic wines with each of the reference compounds (5 levels of concentration covering the concentration ranges expected in wines × 3 repetitions) were used, after checking the absence of significant matrix effects for most of the volatile analyzed by the comparison of the slopes of the regression curves obtained in the synthetic and real wines.

2.3. Wine color and phenolic compounds

Color was determined by measuring absorbance at 420 nm (10-mm cell) using a Beckman Coulter DU-800 spectrophotometer (Fullerton, CA, USA). Total polyphenol index was analyzed as the absorbance at 280 nm directly measured in the wine using a 1-mm cell. The value of the total polyphenol index (TPI) was calculated by multiplying absorbance × 10.

Wines were assayed for total polyphenols (TP) using the Folin–Ciocalteu reagent (Singlenton & Rossi, 1965). Results were expressed as mg of gallic acid per liter. Analysis was carried out in duplicate. Wines were assayed for catechins following the method of Swain & Hillis, 1959, and results were expressed as mg of (+)–catechin per liter. Analysis was carried out in duplicate.

The wine browning potential was determined following the method of Cosme, Ricardo-da-Silva, & Laureano, 2008. Test tubes were filled to 75% with the wine to be tested. Controls were sparged

	Excellent	Very good	Good	Correct	Ordinary	Defective	Eliminated
Visual Aspect	0	1	3	4	6	9	∞
Aroma Intensity	0	2	6	8	12	18	∞
Aroma Quality	0	2	6	8	12	18	∞
Taste Intensity	0	2	6	8	12	18	∞
Taste Quality	0	3	9	12	18	27	∞
Harmony	0	3	9	12	18	27	∞

Fig. 1. Tasting card used by a panel of experts comprised of eight judges in the sensory evaluation of the Albariño white wines.

thoroughly with nitrogen and test samples were sparged with oxygen. All tubes were sealed and maintained at 55 °C for 5 days. After this time, the absorbance at 420 nm was measured in the wines using a 10-mm cell. The browning potential of a wine was calculated as the difference in absorbance between the oxygen-sparged wine and the control wine. Tests were carried out in duplicate.

For the identification and quantification of phenolic compounds, a Waters (Milford, MA, USA) liquid chromatography system equipped with a 2695 Alliance separation module, a 2996 photodiode-array detector (DAD), and a 2475 fluorescence detector was used. Separation was performed on a reversed-phase Waters Nova-Pak C18 (250 mm × 4.6 mm, 4 μm) column at room temperature. A gradient consisting of solvent A (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic acid, 78:20:2, v/v/v) was applied as follows: from 0 to 55 min, 100-20 %A, 0-80 %B, 1 mL/min; from 55 to 65 min, 20-0 %A, 80-0 %B, 0–100% methanol, 1–1.2 mL/min, from 65 to 75 min, 100% methanol, 1.2 mL; and re-equilibration of the column from 75 to 95 min. The detection conditions were: 210–360 nm (DAD); 280 nm and 310 nm for the emission and excitation filters, respectively (fluorescence detector). Identification of chromatographic peaks was achieved by comparing with retention times and UV spectra of phenolic standards. Quantification was carried out by external standard calibration curves. Gallic acid was quantified at 280 nm; hydroxycinnamic acids and their derivatives at 340 nm; and flavan-3-ols and tyrosol by their fluorescence response. Due to the lack of commercial standards, hydroxycinnamic derivatives were quantified using the free acid calibration curve, and procyanidin dimers were quantified using the (+)-catechin calibration curve.

2.4. Sensory analysis

Sensory evaluation of the wines was carried out by a panel of experts comprised of eight judges. The tasting card used was the official Rias Baixas index card (Fig. 1). Wine samples were evaluated at 15 °C. The scores used were penalizing scores, so the better quality wines received a lower score. Six variables (visual examination, aroma intensity, aroma quality, taste intensity, taste quality and harmony) were proposed for assessment, and a scale of 7 categories designed (excellent: 0–7, very good: 8–23, good: 24–44, correct: 45–52, ordinary: 53–78, defective: 79–90, eliminated: >90). The mode of the scores given by the eight tasters was used to arrive at the final score for each parameter corresponding to the sensorial characteristics of wine.

2.5. Statistical analysis

Significant differences among the data obtained from the volatile and phenolic composition of the wines elaborated with different yeasts were estimated by applying analysis of variance (ANOVA). The Tukey least significant differences (LSD) test was used to evaluate the significance of the analysis. The program used was SPSS 16.0 for Windows, version 16.0.1 (Nov. 2007).

3. Results and discussion

3.1. Inoculation of Albariño must with the selected yeast

All the inoculated musts follow similar fermentation kinetics, but the sugar consumption was slower in the control tank (C) than in the other wines (Fig. 2). While the must inoculated with strains 1, 2 and 3 was fully fermented in 25 days, the remaining sugar in the control wine at this time was 67.2% of the initial value, and fermentation lasted until 35 days. These differences in the fermentative activity among the inoculated musts and the control indicate that yeast strains 1, 2, and 3 play a key role in the fermentation. In the must inoculated with yeast 1, the sugar content rapidly decreased, indicating that this strain is better adapted to the must, presenting a higher fermentation activity. The mitochondrial DNA profile

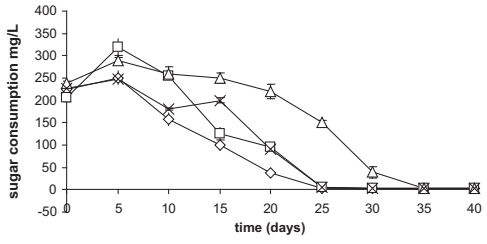

Fig. 2. Measurement of the fermentation progress in the wines inoculated with different yeast strains (1 (◇), 2 (□) and 3 (X)) and the control wine (CW) (Δ), expressed as sugar consumption (mg/L).

showed the percentage of yeast imposition in each fermented must. In all tanks, the percentage of imposition for strains 1, 2, and 3 was between 90% and 100% in all stages of the fermentation. The control must (C) have a heterogeneous yeast population, which included yeast strains 1 and 3 with an average percentage imposition from 43% to 20%, respectively. All wines showed no significant differences ($p < 0.05$) among the chemical parameters determined. The average values were as follows: alcoholic grade 12.2%, total acidity 6.7 g of tartaric acid/L, volatile acidity 0.2 g of acetic acid and pH 3.3.

3.2. Study of the volatile composition of wines

Table 1 shows the volatile profile of the different wines studied. The wines fermented with strain 1 have a significantly higher concentration of terpenes and norisoprenoids than the other wines, with the exception of nerol, in spite of all wines having been elaborated with the same must. Terpenes and norisoprenoids mainly depend on grape varietal characteristic, but can also be influenced by the fermenting yeast and/or by other compounds present in the wine (Ugliano et al., 2006). Geraniol and linalool were the major terpenes in wine 1, and their concentration was higher than in the other wines. Geraniol and linalool are considered to be the most important of the monoterpenes as they are present in greater concentrations and have flavor thresholds lower than others (Pedersen, Capone, Skouroumounis, Pollnitz, & Sefton, 2003). In the wines elaborated with yeast 1, both monoterpenes were found above the perception threshold, 130 μg/L for geraniol (Swiegers, Bartowsky, Henschke, & Pretorius, 2005) and 50 μg/L (Francis & Newton, 2005) for linalool, respectively. Although the mildly acidic conditions of wine can contribute to the release of these compounds during winemaking (Sefton, Francis, & Williams, 1993), it has recently been found that the majority of linalool and geraniol formed during fermentation could be due to enzymatic yeast –driven by the hydrolysis of glycosides (Ugliano & Moio, 2008), demonstrating a potential role of the yeast strain in the terpene concentration of wine.

The concentration of norisoprenoids (α-ionone and β-damascenone) was also significantly higher in the wine elaborated with yeast 1 than in the other wines. As with the monoterpenes, the presence of norisoprenoids is also considered to be a quality factor in these wines, as they are associated with an agreeable scent of tea, fruits, and flowers, mainly rose and violet (Etievant, 1991).

Higher alcohols were quantitatively the largest group of volatile compounds present in the wines. These compounds are produced by yeast metabolism during alcoholic fermentation and their role in wine flavor depends on the types of compounds and their concentrations (Valero, Moyano, Millan, Medina, & Ortega, 2002). Most of these compounds are associated with unpleasant aromas in wines, but although significant differences were found among the strains, the values obtained were below the limit in which they could affect the sensorial properties of the wines. Regarding the alcohols, 2-phenylethanol is associated with a positive floral aroma which has a pleasant rose-like odor (Tosi, Azzolini, Guzzo, & Zapparoli, 2009), but no significant differences were observed between the wines.

Esters are another group of fermentative aromas which are usually associated with fruity descriptors, but in a wine can significantly affect the global aroma (Swiegers et al., 2005). The major ester in the wines was isoamyl acetate, but no significant differences were detected among them. Although similar values were obtained for the other esters determined, significant differences among the wines were obtained for most of these compounds. Concentrations of the fatty acids analyzed (hexanoic and octanoic acids) were close to their perception threshold, which is around 8–9 mg/L (Lambrechs and Pretorious, 2000), and no significant differences were found among strains.

3.3. Phenolic composition of wines

No significant differences were found in wine color -measured as absorbance at 420 nm-among the Albariño wines elaborated with strains 1, 2 and 3 and control wine (Table 2). For these wines, total polyphenols were in the range of 201–214 mg/L. The control

Table 1
Content (mg/L) of each aroma compounds identified in wines (Mean ± SD).

Concentration (mg/L)	Wine 1	Wine 2	Control wine	Wine 3
Terpenes and norisoprenoids				
geraniol	0.17c ± 0.002	0.02a ± 0.001	0.05b ± 0.001	0.05b ± 0.001
linalool	0.06c ± 0.002	0.03b ± 0.002	0.03b ± 0.001	0.02a ± 0.002
nerol	0.01a ± 0.001	0.01a ± 0.002	0.03c ± 0.001	0.02b ± 0.002
α-ionone	0.08c ± 0.002	0.05a ± 0.002	0.06b ± 0.002	0.05a ± 0.001
β-damascenone	0.06c ± 0.002	0.03a ± 0.002	0.03a ± 0.002	0.05b ± 0.003
Alcohols				
Methanol	32.6a ± 2.8	47.2bc ± 3.9	42.9b ± 4.1	56c ± 3.5
Hexanol	0.37b ± 0.01	0.28a ± 0.01	0.50c ± 0.03	0.47c ± 0.03
2-phenylethanol	16.1a ± 3.1	14.3a ± 2.5	13.2a ± 3.7	14.8a ± 2.2
1-propanol	48.2ab ± 3.5	70.8c ± 6.3	62.3bc ± 3.4	41.6a ± 7.6
2-methyl-1-propanol	18.8a ± 2.8	15.2a ± 3.0	12.5a ± 4.5	17.6a ± 3.0
2-methyl-1 butanol	24.3a ± 3.6	24.1a ± 2.8	22.4a ± 3.3	26.3a ± 4.9
3-methyl-1 butanol	108.5bc ± 8.3	115c ± 7.2	107.1abc ± 8.4	83.9a ± 11.3
Esters				
Ethyl butyrate	0.97b ± 0.07	1.22c ± 0.08	0.57a ± 0.09	1.17a ± 0.03
Ethyl hexanoate	0.74b ± 0.005	0.79c ± 0.004	0.80ac ± 0.01	0.76b ± 0.01
Hexyl acetate	0.41a ± 0.003	0.47b ± 0.01	0.55d ± 0.005	0.49c ± 0.01
Butyl acetate	0.54a ± 0.004	0.69d ± 0.005	0.55ab ± 0.01	0.61c ± 0.005
Isobutyl acetate	0.58a ± 0.004	0.63b ± 0.005	0.58a ± 0.005	0.57a ± 0.003
Isoamyl acetate	2.28a ± 0.72	2.84a ± 0.51	3.71a ± 0.83	3.64a ± 0.62
Ethyl octanoate	0.69b ± 0.03	0.68b ± 0.03	0.46a ± 0.03	0.64b ± 0.04
Ethyl decanoate	0.11a ± 0.02	0.28b ± 0.03	0.12a ± 0.03	0.09a ± 0.01
Acids				
Hexanoic acid	10.59a ± 2.14	11.72a ± 1.34	15.54a ± 3.54	14.03a ± 2.12
Octanoic acid	8.72a ± 1.03	10.22a ± 0.97	9.70a ± 0.91	10.81a ± 1.12

a, b, c, d- Same letter in the same row indicates absence of significant differences ($p < 0.05$).

Table 2
Color, total phenolic determinations and browning potential of wines (Mean ± SD).

	Wine 1	Wine 2	Control wine	Wine 3
Color				
(Abs 420 nm)	0.0766a ± 0.003	0.0726a ± 0.004	0.0741a ± 0.001	0.0764a ± 0.001
TPI				
(Abs 280 nm)	6.71a ± 0.16	6.93a ± 0.27	6.37b ± 0.17	6.84a ± 0.06
Total polyphenols				
(mg gallic acid/L)	205ab ± 4	201b ± 10	204ab ± 4	214a ± 2
Total catechins				
(mg (+)-catechin/L)	38.7c ± 1	44b ± 1	47.6a ± 2.3	46.3ab ± 0.1
Browning potential				
(ΔAbs 420 nm)	0.0116c ± 0.005	0.0443a ± 0.002	0.0285b ± 0.006	0.0357ab ± 0.003

a, b, c- Same letter in the same row indicates absence of significant differences ($p < 0.05$).

wine and the wine fermented with strain 3 showed higher contents of total catechins – the main phenolic group- than the others, and the wine fermented with strain 1 presented a significantly lower content (Table 2). The browning potential, which is an indicator of wine oxidability, was significantly lower for the wine fermented with strain 1 (Table 2), indicating that this wine would be more stable to oxidation.

A total of thirteen compounds were identified and quantified in the Albariño wines by LC-DAD-fluorescence analysis. They corresponded to hydroxybenzoic acids (gallic acid), phenolic alcohols (tyrosol), hydroxycinnamic acids and derivatives (*trans*- and *cis*-caftaric acid, *trans*- and *cis*-cutaric acid, caffeic acid and a caffeic acid derivative), monomeric flavan-3-ols [(+)-catechin and (−)-epicatechin], and procyanidins [B1 or epicatechin-(4β→8)-catechin, B3 or catechin-(4α→8)-catechin, and B2 or epicatechin-(4β→8)-epicatechin] (Table 3). B1 and B3 were not totally resolved and were quantified together. In a recent study, de Quiros, Lage-Yusti, & Lopez-Hernandez, 2009, reported eleven phenolic compounds in Albariño wines. Most of these coincided with the ones identified in our study, although some flavonols and stilbene derivatives were also detected.

The wine fermented with strain 3 showed a significantly higher content of gallic acid than the other Albariño wines (Table 3). This acid is the only hydroxybenzoic acid that has been formally identified in its native state in grapes, and is found in the solid parts of the berry either in free form or as the flavanol ester (i.e., epicatechin-3-*O*-gallate) (Su & Singleton, 1969).

Tyrosol content was also affected by the yeast strain used. Wines elaborated with strain 1 and the control wine showed significantly higher values than wines elaborated with strains 2 and 3 (Table 3). Tyrosol is formed during yeast fermentation from tyrosine (3-(4-hydroxyphenyl)-alanine). Previous studies have also shown significant differences in tyrosol content among wines inoculated with different yeast strains (Monagas, Gomez-Cordoves, & Bartolome, 2007).

In relation to hydroxycinnamic acids and derivatives, the wine elaborated with strain 1 showed the lowest content for all the individual compounds whereas the wines elaborated with strain 2 and the control wine showed the highest contents in most cases (Table 3). When the sum of the individual contents was considered, the wine elaborated with strains 2 (30.1 mg/L) and 3 (29.0 mg/L) and the control wine (29.0 mg/L) showed significantly higher values than the wine elaborated with strain 1 (26.9 mg/L) (data not shown). The hydroxycinnamic esters caffeoyltartaric (caftaric), *p*-coumaroyltartaric (cutaric) and feruloyltartaric (fertaric) acids are present in the grape skin and pulp. During must fermentation, hydrolysis occurs and free hydroxycinnamic acids can be found in wine (Singleton, Timberlake, & Lea, 1978) and the yeast strain may condition this hydrolytic activity (Monagas et al., 2007).

Similarly to hydroxycinnamic acids and derivatives, the wine elaborated with strain 1 showed the lowest contents of all the monomeric and dimeric flavan-3-ols, whereas the wine elaborated with strain 2 and the control wine C showed the highest contents in most cases (Table 3). To summarize, flavan-3-ol content was in the order: wine elaborated with strain 2 (33.1 mg/L) > control wine (31.9 mg/L) > wine elaborated with strain 3 (31.8 mg/L) > wine elaborated with strain 1 (30.0 mg/L) (data not shown). Flavan-3-ols, especially procyanidins, are largely responsible for the astringency and bitterness of the wine (Ribichaud & Noble, 1990) and also participate in haze formation and interactions with proteins (Cheynier & Ricardo da Silva, 1991; Ricardo da Silva et al., 1991), as well as in numerous condensation reactions during wine maturation and aging (Haslam, 1980).

The lowest hydroxycinnamic acids and flavan-3-ols contents were found for wine elaborated with strain 1 and this wine also showed the lowest browning potential, as described above (Table 3). It is known that both types of phenolic compounds make a major contribution to the browning phenomena in white wines (Li, Guo, & Wang, 2008).

Table 3
Content (mg/L) of each phenolic compound identified in wines (Mean ± SD).

	Wine A	Wine B	Wine C	Wine D
Gallic acid	5.15b ± 0.14	5.14b ± 0.16	5.18b ± 0.18	5.64a ± 0.05
trans-Caftaric acid	10.5b ± 0.3	11.8a ± 0.6	12.0a ± 0.5	12.0a ± 0.1
cis-Caftaric acid	5.21b ± 0.55	6.11a ± 0.85	4.86b ± 0.30	4.99b ± 0.12
cis-Cutaric acid	3.20b ± 0.04	3.42a ± 0.15	3.28ab ± 0.14	3.30ab ± 0.02
trans-Cutaric acid	3.94c ± 0.14	4.51a ± 0.30	4.54a ± 0.24	4.43ab ± 0.23
Caftaric acid derivative	2.07b ± 0.05	2.36a ± 0.11	2.36a ± 0.10	2.31a ± 0.03
Caffeic acid	1.97ab ± 0.03	1.89b ± 0.07	2.04a ± 0.04	2.00a ± 0.02
Procyanidins B1+B3	1.35b ± 0.08	1.51a ± 0.07	1.52a ± 0.05	1.43ab ± 0.04
Tyrosol	10.0a ± 0.3	8.8b ± 0.3	10.0a ± 0.04	8.5b ± 0.1
(+)-Catechin	17.0c ± 0.7	19.1a ± 0.6	18.0b ± 0.4	18.5ab ± 0.4
Procyanidin B2	1.33c ± 0.15	1.63a ± 0.06	1.63a ± 0.07	1.56ab ± 0.05
(−)-Epicatechin	10.3b ± 0.4	10.9a ± 0.1	10.8a ± 0.2	10.3b ± 0.2

a, b, c- Same letter in the same row indicates absence of significant differences ($p < 0.05$).

Table 4
Sensory analysis of the different wines. Results are expressed as the average score obtained from eight judges (Mean ± SD).

Attributes	Wine 1	Wine 2	Control wine	Wine 3
Visual aspect(0–9)	3.6a ± 0.5	3.9a ± 0.3	3.6a ± 0.5	3.7a ± 0.4
Aroma intensity(0–18)	2.5a ± 1.3	7.2b ± 1.0	7.5b ± 0.9	7.5b ± 0.9
Aroma quality(0–18)	3.0a ± 1.7	7.7b ± 0.7	7.5b ± 0.9	7b ± 1.0
Taste intensity(0–18)	2.5a ± 1.3	6.5b ± 0.9	7.7b ± 0.7	6.2b ± 0.7
Taste quality(0–27)	3.7a ± 2.0	9.4b ± 1.0	11.2b ± 1.3	10.1b ± 1.4
Harmony(0–27)	3.7a ± 2.0	9.4b ± 1.0	12c ± 0.0	9.7b ± 1.3
Total[a]	19.4	44.2	49.9	44.5

a, b, c- Same letter in the same row indicates absence of significant differences ($p < 0.05$).
[a] 0–7 = excellent; 8–23 = very good; 24–44 = good; 45–62 = correct; 63–78 = regular; 79–90 = inadequate; and >90 = eliminated.

3.4. Sensory analysis

Table 4 shows the average scores for each wine in the sensory evaluation. The tasters gave wine 1 a lower score than the other wines. Because the scoring used on the card is based on a penalizing system, the result indicates that the tasters consider this wine to have a better quality. As visual aspect was similar in all wines, attributes related to aroma and taste were responsible for the differences observed. As described above, wine elaborated with yeast 1 presented a higher concentration of terpenes and norisoprenoids than the other wines. Four of these compounds (geraniol, linalool, α-ionone and β-damascenone) were found in concentrations above the odor threshold (Sanchez Palomo, Diaz-Maroto, Gonzalez Viñas, Soriano-Perez, & Perez-Coello, 2007; Swiegers et al., 2005), so they could be responsible for the differences observed among wines. The lower amount of procyanidins present in wine elaborated with strain 1 could also contribute to its higher acceptability, but in both cases this must be investigated further. On the other hand, the control wine obtained by "spontaneous" fermentation was the given the poorest evaluation by tasters, and wines 2 and 3 were considered as very similar.

4. Conclusions

Locally-selected yeast can affect the fermentation and influence the volatile and phenolic profile of white wines elaborated with the same Albariño must when they are used as single inoculum. Although wines elaborated with "spontaneous" fermentation and yeast strains 2 and 3 were considered correct by tasters, only the wine elaborated with the strain 1 was qualified as very good. The most interesting chemical attributes of the wine elaborated with strain 1 were its high concentrations of terpenes and norisoprenoids, which have been previously found to be key compounds in determining the fruity and fresh character of these wines. Also, the wine elaborated with strain 1 had a lower concentration of flavan-3-ols, closely related with the astringency and bitterness of the wine and the lowest browning potential, which could help to conserve some of its sensorial properties over time. Different aspects of its contribution to the global properties of these white wines are currently being investigated in our laboratory.

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CHAPTER 2

Influence of Yeast Mannoproteins in the Aroma Improvement of White Wines.

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CAPÍTULO 2

INFLUENCIA DE LAS MANOPROTEÍNAS DE LEVADURA EN LA MEJORA DEL AROMA DE VINOS DE DE ALBARIÑO DE LA D.O. RÍAS BAIXAS

OBJETIVO

Una vez seleccionada la cepa más idónea para llevar a cabo la vinificación controlada de mostos de *Vitis vinifera* cv. Albariño de la D.O. Rías Baixas se comprobó que, entre otros aspectos, los vinos obtenidos con esta cepa eran más ricos en manoproteínas y en aromas varietales, por lo que se decidió estudiar si existía alguna relación entre la concentración de manoproteínas de estos vinos y su perfil aromático, siendo este el objetivo principal del presente capítulo.

PLAN DE TRABAJO

Para el desarrollo de este objetivo se llevaron a cabo las siguientes tareas:

1. Aislamiento, hidrólisis y cuantificación de las manoproteínas en vinos elaborados en bodega por vinificaciones controladas a escala piloto (30 L) de mostos de *Vitis vinifera* cv. Albariño de la D.O. Rías Baixas, llevadas a cabo con las cepas seleccionadas de *S. cerevisiae* utilizadas en este estudio.
2. Identificación y cuantificación de los compuestos volátiles de los vinos estudiados.
3. Estudio de la capacidad de retención *in vitro* de dos aromas varietales (geraniol y linalool) por parte de la fracción coloidal rica en manoproteínas liberada por la cepa de *S. cerevisiae* seleccionada para la vinificación controlada de mostos de *Vitis vinifera* cv. Albariño de la D.O. Rías Baixas.

4. Análisis sensorial de los vinos obtenidos y estudio de la relación entre las manoproteínas liberadas y la composición volátil.

RESUMEN

En estudios previos se ha demostrado que las manoproteínas liberadas por las levaduras durante la fermentación y la autolisis pueden estar involucradas en la retención de algunos compuestos del aroma. En el presente trabajo, una vez determinado el efecto de la cepa de levadura en la composición químicos (compuestos aromáticos y fenólicos), así como en la propiedades sensoriales de vinos Albariño, se llevó a cabo el estudio de las manoproteínas liberadas al vino por las cepas autóctonas seleccionadas (*S. cerevisiae* 1, 2, y 3) y su relación con los compuestos volátiles y el perfil sensorial de los vinos blancos obtenidos. Para llevar a cabo este estudio, mostos de la variedad Albariño se inocularon con las cepas seleccionadas. La concentración de manoproteínas se determinó a partir del contenido en coloides proteicos y manosa polimérica en los vinos, y resultó ser significativamente mayor en los vinos inoculados con la cepa de levadura *S. cerevisiae* 1. El análisis de la composición aromática de los vinos obtenidos reveló que en las fermentaciones inoculadas con las cepas autóctonas tenían una concentración mayor que la fermentación no inoculada (control). En el caso de los aromas varietales, terpenos y norisoprenoides, los vinos elaborados con la cepa de levadura *S. cerevisiae* 1 presentaron una mayor concentración de casi todos los compuestos analizados. Con el objetivo de comprobar si las manoproteínas liberadas por esta cepa estaban relacionadas con la mayor concentración de compuestos varietales en estos vinos se diseñó un experimento *in vitro* utilizando un medio vínico modelo. Se aisló la fracción coloidal de los vinos inoculados y se suspendió en el vino modelo. La solución obtenida se inoculó con patrones puros de geraniol y linalol en una concentración final de 10 mg/L, estudiándose la capacidad de retención de estos compuestos por parte de la fracción coloidal de cada una de las cepas.

Los resultados obtenidos demostraron que la fracción coloidal correspondiente al vino elaborado con la cepa *S. cerevisiae* 1 retuvo un mayor porcentaje de estos compuestos que la fracción coloidal de los otros vinos inoculados y del vino obtenido por fermentación espontánea. Estos resultados coinciden con la presencia en la fracción coloidal del vino elaborado por *S. cerevisiae* 1 de una mayor cantidad de manoproteínas, e indican una posible interacción de las manoproteínas con los compuestos aromáticos del vino, incrementando así su persistencia y en consecuencia su percepción sensorial. Precisamente, el análisis sensorial de los vinos obtenidos, realizado mediante un sistema de evaluación de penalización en el que los mejores vinos recibían las calificaciones más bajas, demostró que los vinos elaborados con la cepa de levadura *S.cerevisiae* 1 fueron los que obtuvieron menor calificación en todos los parámetros analizados (aroma, sabor y armonía).

Influence of Yeast Mannoproteins in the Aroma Improvement of White Wines

M. Juega, Y.P. Nunez, A.V. Carrascosa, and A.J. Martinez-Rodriguez

Abstract: In the present work, 3 different yeast strains (1, 2, and 3) were used to elaborate white wines using Albariño must. The concentration of polymeric mannose was determined using a method based on the mannoprotein precipitation, hydrolysis and analysis of sylylated mannose derivatives by gas chromatography. Wines elaborated with the strain 1 (W1) presented a higher mannoprotein concentration than the other wines. The analysis of the volatile composition of wines showed significant differences ($P < 0.05$) among them, being W1 which presented the highest concentration of aroma compounds, mainly terpens and norisoprenoids. The sensorial analysis of wines also showed that W1 had the best quality. The results obtained from this work demonstrate that mannoproteins could be involved in the behavior observed. Some evidences were obtained using a model wine, where 2 major terpens in W1 were preferentially retained by the colloids rich in mannoproteins released by strain 1.

Keywords: Albariño, aroma, mannoproteins, white wines, yeast

Practical Application: White wines elaborated with yeast strains overproducing mannoproteins could have better quality than others. Mannoproteins could contribute to aroma enhancement of Albariño white wines

Introduction

Mannoproteins are among the main components of yeast cell walls. The outer layer of the wall contains β-1,6 glucan (5% to 10%) and mannoproteins (35% to 40%), covalently bound to β-1,3 glucan, either directly or via a fragment of β 1 to 6 glucan (Caridi 2006). These mannoproteins present a high degree of glycosylation (between 50% and 95%) with α-mannose binding units (α-1,6; α-1,2 and α-1,3) (Klis and others 2002). Over the past few years there has been increasing interest in the possible enological applications of mannoproteins and their association with improved wine quality. Some scientific studies have established a relationship between the presence in, and/or addition of mannoproteins to wine and reduced tartaric acid precipitation (Moine-Ledoux and Dubourdieu 2002; Bowyer and Moine-Ledoux 2007) and protein haze (Dupin and others 2000; Gonzalez-Ramos and others 2008). Mannoproteins have also been reported to help in the stabilization of color and to reduce astringency in red wines (Escot and others 2001; Guadalupe and others 2010), and have also been associated with the fixation of some aromatic wine components (Lubbers and others 1994; Comuzzo and others 2011) and the improved quality of sparkling wine foam (Nunez and others 2006).

The white variety *Vitis vinifera* Albariño is a traditional Galician grape and produces white wines with distinctive fruity and flowery aromas. The aromatic composition of these wines has been shown to be determined by several variables, such as geographical origin (Falque and others 2008) and/or the yeast strain used (Vilanova and Massneuf-Pomarede 2005; Carrascosa and others 2012). However, it has not yet been studied whether mannoproteins released by yeasts play a role in determining the quality of these wines, especially their aromatic composition, which is one of their most distinguishing features. Some studies have analysed the interaction between different aromatic compounds and yeast walls or mannoproteins (Lubbers and others 1994; Chalier and others 2007; Comuzzo and others 2011; Cortes and Blanco 2011), but most have been carried out using model systems, so their real impact in such a complex medium as wine is unknown.

In this work, 4 white wines have been produced using Albariño must and 3 different yeast strains. A wine obtained from noninoculated fermentation of the Albariño must was used as an experimental control. In all cases, musts were fermented for approximately 30 d, and the mannoprotein and aromatic composition of the wines were determined, in an attempt to establish a possible association between the 2 parameters and how they influence the quality of the final wines.

Material and Methods

Must, yeast, and fermentation conditions

The must used in this study was from *Vitis vinifera* cv. Albariño grapes and was supplied by the winery Terras Gauda (Galicia, Spain).

To carry out the fermentation, 3 different yeast strains (1, 2, and 3) were used. These strains were isolated from different vinification processes in the wine cellar from 2 previous vintages produced without yeast inoculation. In all cases, they were the predominant yeast at the end of the fermentation. Microvinifications (1L) were previously done in the laboratory to check the fermentative capacity of the selected yeast at 18 °C (Quiros and others 2011) and the following chemical characteristics of the wines (alcoholic grade, total acidity, volatile acidity, and pH). All the parameters were

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determined by the European Commission methods (EC 1990). For the experiments developed in the cellar, the must was inoculated with yeast and fermented in 30 L stainless steel tanks. Temperature was set to 18 °C. A control wine (WC) was prepared by fermenting the must with its own yeast. In all cases, experiments were carried out in triplicate. Predominance of the selected yeast in the fermentation tanks of the inoculated wines was verified by studying the mitochondrial DNA profile in different stages of the fermentation (initial phase: 3 d, middle fermentation: 15 d, and at the end of fermentation: 25 d), following the method described by Querol and others 1992. Fermentation was followed by the sugar consumption in each tank and the reducing sugar (Flanzy 2003) during fermentation was determined until 40 d. When fermentation was complete in each case, 250 mL samples were taken from each tank. Samples were cleared by centrifugation at 1800 × *g* 15 min, and were used for the determination of mannoproteins and volatile compounds.

Precipitation, hidrolysis, and cuantification of mannoproteins

The procedure described by Segarra and others (1995) was used for the isolation of colloidal fraction containing mannoproteins. Fourty mL of ethanol (96% v/v) and 400 μL HCL (1N) were added to 8 mL of wine. After 18 h of incubation at 22 °C, the tubes were centrifuged (1800 × *g*, 20 min), after which the supernatant was discarded and the pellet was washed 3 times in ethanol (96%, v/v). For the determination of the sugar composition of mannoproteins, the samples obtained were hydrolyzed at 100 °C for 24 h in a closed vial containing 1 mL of 2 M trifluoroacetic acid and 0.5 mL myo-inositol (0.1% w/v, internal standard) solution. After hydrolysis, the mixture was evaporated to dryness under vacuum.

The dried hydrolyzed residue was silylated following the procedure described by Nunez and others (2006). Briefly, the sample was dissolved in 100 mL of anhydrous pyridine, and 100 μL of trimethylsilylimidazole, 100 μL of trimethylclorosilane, 100 μL of n-hexane, and 200 μL of deionized water were sequentially added, shaking during each step. Trimethyilsilyl derivatives (1μL) were analysed on a Hewlett-Packard 6890 Chromatograph (Palo Alto, Calif., U.S.A.), equipped with a flame ionization detector (FID) and split/splitless injector. Samples were injected on a Carbowax 20M column (30 m × 0.25 mm) coated with a stationary phase of 0.25 mm thickness. Temperatures were as follows: injector and detector, 220 °C; oven, held at 175 °C for 15 min, then increasing 15 °C/min to 200 °C during 13 min and finally programmed at 30 °C/min to 270 °C during 20 min. The carrier gas was helium (10 psi, split 1/15). Response factors were calculated with a series of pure standards at different concentrations using myo-inositol as internal standard. The identification of the mannose present in the samples was carried out by comparing the retention time of the peaks with those of pure standard. Each sample was analyzed by triplicate. Results were expressed as mg/L of polymeric mannose in the wine. The concentration of protein moieties was determined following the Bradford method (Bradford 1976), based in the reaction of the protein with the Coomasie blue G-250. Absorbance was measured at 595 nm 15 min after the addition of the reactive. The results were expressed in mg of bovine seroalbumine (BSA)/L.

Volatile compounds

The analysis of the major volatile compounds (higher alcohols) was performed by direct injection in a Hewlett-Packard 5890 series II gas chromatograph equipped with flame ionization detection (FID) and a split/splitless injector. Separations were carried out on a Chrompack CP-WAX 57 CB fused silica capillary column (50 m × 0.25 mm i.d.) coated with a 0.25-μm thick polyethylene glycol stationary phase (Varian, Houten, The Netherlands) and the injector and detector temperatures were 220 °C. The temperature program was as follows: initial temperature 40 °C (10 min hold) and ramps of 5 °C/min to 200 °C and 20 °C/min to 210 °C during 20 min. The carrier gas was helium (15 psi). A total of 50 μL of 3-pentanol (6 mg/mL 10% ethanol) was added as internal standard to 10 mL of wine, and 2 μL of wine with the internal standard was injected in the split mode. A ChemStation data system (HP 3365 series II, v. A.03.21) was used for data acquisition and processing. The compounds determined by this method were methanol, 2-phenylethanol, 1-propanol, 2-methyl-1-propanol, and 2- and 3-methyl-1-butanol.

Minor volatile compounds (esters and acetates) were analyzed by gas chromatography (GC) of the headspace extract obtained with a 100 μm poly-dimethylsiloxane coated fused silica fiber (Supelco, Bellefonte, Pa., U.S.A.), in the conditions described by Pozo-Bayon and others (2001), using methyl nonanoate as internal standard. The compounds determined by this method were ethyl butyrate, ethyl hexanoate, hexyl acetate, butyl acetate, isobutyl acetate, isoamyl acetate, ethyl octanoate, and ethyl decanoate. The temperature of both injector and detector was 250 °C. The temperature program was as follows: initial temperature 70 °C and ramps of 5 °C/min to 200 °C and 3 °C/min to 215 °C. The carrier gas was helium (15 psi).

Free terpenes and norisoprenoids were fractionated by selective retention on SepPak Vac C-18, according to the procedure described by Vilanova and Sieiro (2006). The free fraction was eluted with pentane dichloromethane (2:1, 10 mL) and the eluate was dried over anhydrous sodium sulphate and concentrated to 0.5 mL, by evaporation with a stream of nitrogen, before GC analysis. The conditions used for chromatographic analysis were: injector temperature (250 °C), detector temperature (260 °C), injection type (Splitless, 30 s), and injection size (1 μL). The temperature program was as follows: initial temperature 70 °C (7 min hold) and ramps of 2 °C/min to 120 °C and 3 °C/min to 215 °C during 25 min. The carrier gas was helium (14.5 psi). The compounds determined by this methodology were geraniol, linalool, nerol, β-ionone, and β-damascenone, using 3-octanol as internal standard.

In all cases, peak identities were assigned by comparing the relative retention times of the internal standard, with those of the standards of analytical quality, of over 99% purity, from Sigma-Aldrich (St. Louis, MO, USA). For quantification purposes, the relative area was obtained as the chromatographic peak area of each aroma compound divided by the area of the internal standard. Calibration curves in synthetic wines with each of the reference compounds (5 levels of concentration covering the concentration ranges expected in wines × 3 repetitions) were used, after checking the absence of significant matrix effects for most of the volatile compounds analyzed by the comparison of the slopes of the regression curves obtained in the synthetic and real wines.

Determination of geraniol and linalool retention by mannoproteins in a model wine

A hydroalcoholic solution with 10% alcohol (v/v) was used as a model wine. The pH was adjusted to 3 with 0.1 M sodium hydroxide. The mannoprotein-rich colloidal fractions isolated from each of the inoculated wines (W1, W2, and W3) and from

the control wine (WC) were washed and dissolved in 5 mL of the model wine. The samples obtained were inoculated with pure standards of geraniol or linalool (Sigma-Aldrich), in a final proportion of 10 mg/L. The mannoprotein retention capacity of volatile compounds was studied by static headspace analysis, following the methodology described by Chalier and others (2007). The compounds were analyzed by GC of the headspace extract obtained with a 100 μm poly-dimethylsiloxane coated fused silica fibre (Supelco). The results were expressed as a percentage of the mannoproteins retention index (RI) for the volatile compounds compared to the RI for volatile compounds dissolved in model wine without the presence of mannoproteins.

Sensory analysis

Sensory evaluation of the wines was carried out by a panel of experts comprising 8 judges. The tasting card used was the official Rias Baixas index card (Carrascosa and others 2012). Wine samples were evaluated at 15° C. The scores used were penalizing scores, so the better quality wines received a lower score. Six variables (visual examination, aroma intensity, aroma quality, taste intensity, taste quality, and harmony) were proposed for assessment, and a scale of 7 categories designed (excellent: 0 to 7, very good: 8 to 23, good: 24 to 44, correct: 45 to 52, ordinary: 53 to 78, defective: 79 to 90, eliminated: >90). The mode of the scores given by the 8 tasters was used to arrive at the final score for each parameter corresponding to the sensorial characteristics of wine.

Statistical analysis

Significant differences among the data obtained from the mannoprotein and volatile composition of the wines were estimated by applying analysis of variance (ANOVA). The Tukey least significant differences (LSD) test was used to evaluate the significance of the analysis. The program used was SPSS 16.0 for Windows, version 16.0.1 (Nov. 2007)

Results and Discussion

Elaboration of the different wines with selected yeasts and determination of the mannoprotein concentration

In all inoculated wines (W1, W2, and W3) the selected strain was found to prevail during the fermentation (data not presented here). No significant differences were found among the chemical parameters determined in the different wines ($P < 0.05$), including the control wine (WC). The average ethanol content in all wines was 12.4% ± 0.18 (v/v). Titratable acidity of wines was 6.5 ± 0.3 g/L, expressed as tartaric acid, and volatile acidity was 0.4 ± 0.01 g/L, expressed as acetic acid; pH was 3.3 ± 0.1. All these values are within the normal ranges found for these wines and show that the vinification was satisfactory.

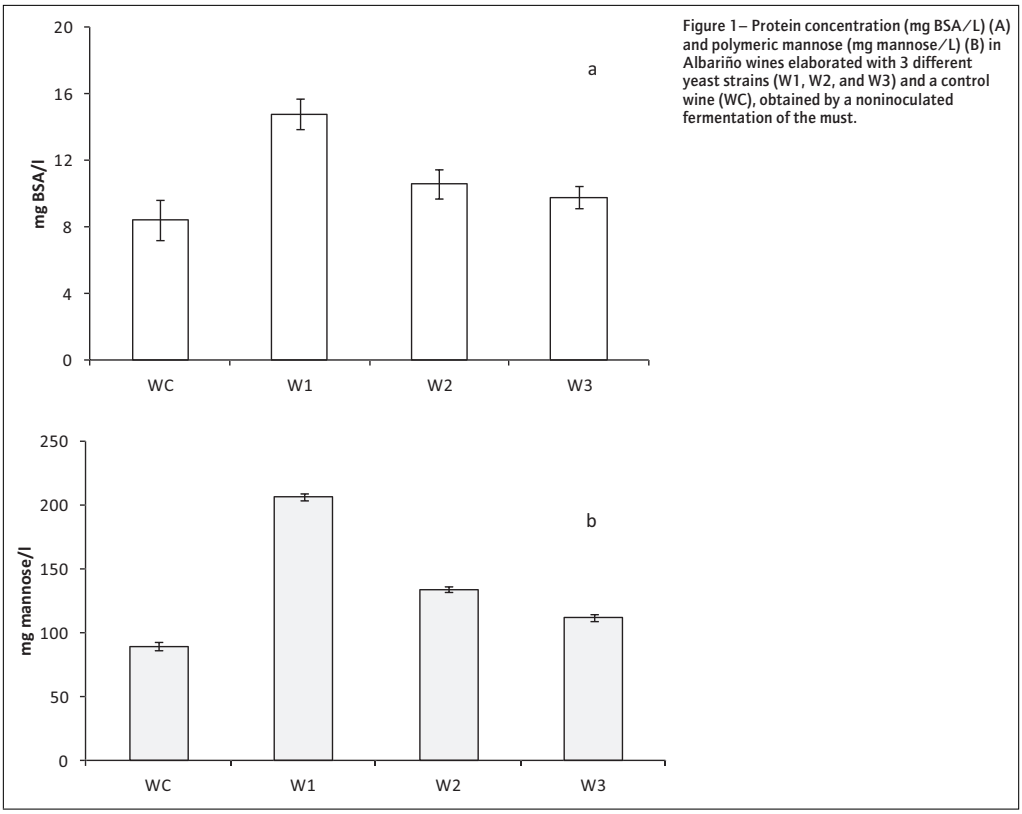

Figure 1– Protein concentration (mg BSA/L) (A) and polymeric mannose (mg mannose/L) (B) in Albariño wines elaborated with 3 different yeast strains (W1, W2, and W3) and a control wine (WC), obtained by a noninoculated fermentation of the must.

Figure 1 shows the quantity of proteins and polymeric mannose in the colloidal fraction present in each wine obtained. In all cases, wines inoculated with the selected strains (W1, W2, and W3) presented a higher concentration of mannoproteins than the noninoculated wine (WC). Wines produced with strain 1 (W1) presented higher contents of proteins (Figure 1a) and polymeric mannose (Figure 1b), which was 35% higher than W2, the second highest. Similar results were obtained in experiments carried out with musts from 2 different harvests (data not shown), suggesting that this strain's ability to release more mannoproteins to the wine is independent of other variables associated with the harvest.

The yeast strain is one of the main factors that control the amount of mannoproteins released during winemaking (Dupin and others 2000; Escot and others 2001; Giovani and others 2010). Therefore, there is increasing interest in selection and development of wine yeast strains with this capability. In the case of Albariño white wines, it has been demonstrated that the yeast strain could be related with the aroma composition of the wine (Vilanova and Massneuf-Pomarede 2005; Carrascosa and others 2012). However, there is no evidence for the effect of mannoproteins on the aromatic quality of Albariño white wines, which is their most distinguishing character.

Analysis of the volatile composition of the wines

Figure 2 shows the results obtained for the determination of the main aromatic compounds detected in the different wines. The majority compounds found corresponded to higher alcohols (Figure 2a) and differences were significant among the wines ($P < 0.05$) for some of the compounds studied. Their concentrations ranged between 12.5 and 115 mg/L, but were below the threshold of 300 mg/L in all cases, considered as the level at which they can begin to negatively affect wine aroma (Flanzy 2003). The alcohol isoamylic 3-methyl-1 butanol was the main compound in all the wines, accounting for 84 mg/L of W3 to 115 mg/L of W2. This compound has been reported to be partially responsible for the fruity aroma of Galician white wines of the Treixadura variety (Cortes and Blanco 2011), and is also one of the most active odorants in Riesling white wine varieties (Komes and others 2006). Another alcohol associated with these types of wines and the presence of a positive floral aroma is 2-phenyl-ethanol, which has an agreeable rose-like odour (Vilanova and others 2007). However, no significant differences in this compound were found among the wines studied.

The majority ester present in the wines was isoamyl acetate, which was found in a concentration ranging from 2.28 mg/L in wine W1 to 3.71 mg/L in WC (Figure 2b). This compound has been described to contribute to the aroma quality of young white wines (Masa and Vilanova 2008; Losada and others 2011). Amongst the esters present, only ethyl octanoate was found at a higher concentration in W1, but this was not significantly different to concentrations determined in W2 and W3.

The analysis of terpenes and norisoprenoids (Figure 2c) showed that wines W1 presented a significantly higher concentration ($P < 0.01$) of the compounds analysed, except for nerol, which had the highest concentration in the control wine (WC). Terpenes and norisoprenoids are the most characteristic aromatic compounds of the Albariño grape variety and are associated with the fresh, fruity, and floral characteristics of these wines (Carrascosa and others 2012). Geraniol was the major terpene in W1 and reached a concentration of 0.19 mg/L, which was almost 10 times higher than in the other wines. This concentration is above the perception threshold described for this compound (0.13 mg/L), indicating that it could have a positive influence on aroma quality (Swiegers and others 2005). Similarly, the concentration of linalool in W1 (0.06 mg/L) was at least twice that of the remaining wines and, this value is also higher than the perception threshold defined for this compound (0.05 mg/L) (Vilanova and others 2007). The concentration of norisoprenoids (β-ionone and β-damascenone) was also significantly higher in W1 than in the remaining wines. These compounds could also contribute to the fruity or floral character of these wines, presenting very low detection thresholds (0.05 to 0.09 μg/L) (Gomez-Miguez and others 2007), lower than the values found in W1.

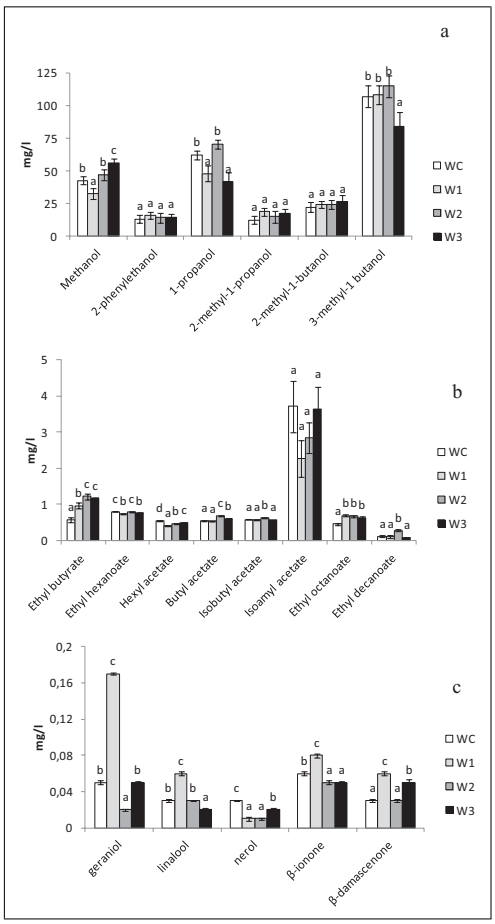

Figure 2– Concentration (mg/L) of the aroma compounds (A) alcohols, (B) esters, and (C) terpenes and norisoprenoids in the different wines (W1, W2, and W3) and the control wine (WC). The same letter means no significant differences ($P > 0.05$) between wines.

Determination of the mannoprotein aroma retention capacity

In an attempt to determine the aromatic compound retention capacity of mannoprotein-rich colloids released in wine W1, we spiked a hydroalcoholic solution with a known concentration of geraniol and linalool, the major compounds found in W1. Several experimental samples were prepared by inoculating them with the colloidal fraction obtained from each wine (MW1, MW2, and MW3) and the control wine (MWC). The results obtained are presented in Table 1. These results show that the colloidal fraction in wine W1 retained the highest percentage of geraniol and linalool compared with results obtained for other wines and the control. RI was higher for geraniol than for linalool. This could be explained by interactions between the colloidal fraction and some aromatic compounds being preferentially established with the less polar compounds, as described previously by other authors (Lubbers and others 1994; Comuzzo and others 2011).

These results agree with the fact that the concentration of mannoproteins in the colloidal fraction is higher in wine W1 than in the other wines studied, suggesting that they could possibly be involved in the retention observed for some aromatic compounds. Moreover, significant differences were not observed either in the total amount of colloids obtained for wines W1 and W2 (data not showed), which once again supports a key role for mannoproteins in the observed behavior.

Some aromatic compounds are known to be able to interact with either polysaccharides or proteins, and there is some previous evidence that mannoproteins can affect the aromatic composition of a wine. Chalier and others (2007) found evidence for an interaction between mannoproteins secreted by yeasts at average concentrations found in wines (150 mg/L) and different aromatic compounds, including ethyl hexanoate and β-ionone. They observed that mannoproteins could help to reduce the volatility of aromatic compounds by more than 80%. Recently, Cortes and Blanco (2011) and Carrascosa and others (2012) have confirmed that the interaction of some aromatic compounds with the yeast cell wall depends on the yeast strain used, and found an association between the use of local yeasts and the aromatic quality of a wine. On the other hand, Comuzzo and others (2011), found that the retention of aromas by mannoprotein-rich colloidal extracts is a complex phenomenon which involves several variables (pH, yeast strain, temperature, interaction with other matrix components, and so on). These authors suggest that this phenomenon may have practical implications for the wine's aromatic quality, because some of these compounds are retained by colloidal substances such as mannoproteins at room temperature, while during the tasting carried out at mouth temperature, many of these aromas are released and could be perceivable at a retronasal level.

Table 1 – Comparative analysis of the retention capacity of geraniol and linalool by mannoprotein extracts of different Albariño wines (MW1–3) and the control wine (MWC). Results are expressed in% as retention index (RI) of volatile compound by the mannoproteins.

	RI (%)	
Samples	Geraniol	Linalool
MW1	18 ± 2.3	7.2 ± 0.6
MW2	2.2 ± 0.6	1.3 ± 0.2
MW3	4.1 ± 0.2	0.8 ± 0.1
MWC	0.36 ± 0.0	0.1 ± 0.0

Sensory analysis

A sensory analysis was carried out in order to establish the practical significance of the results obtained. The results are shown in Figure 3, which depict the "spider-web" diagrams for the average scores of the analyzed wines. It can be observed that the chemical composition of the wines influences the behavior observed during the sensorial analysis. The scoring used on the card is based on a penalizing system, therefore the best wines are those with the lowest scores. The total score of the wines was between 19.4 and 50, which according to the scoring card used, indicates that all the wines obtained are of good quality. The best score was obtained by wine W1, which was considered by the tasters as the one with the best quality. At the other extreme, wine WC obtained the worst score, reflecting a positive relationship between the selection and inoculation of strains for fermentation and wine quality. Apart from the appearance, for which no difference was found among the wines, wine W1 obtained a lower score for all the parameters analyzed (aroma, taste, and harmony), thus indicating a better quality. This wine presented a significantly higher concentration of terpenes and norisoprenoids (linalool, geraniol, β-ionone

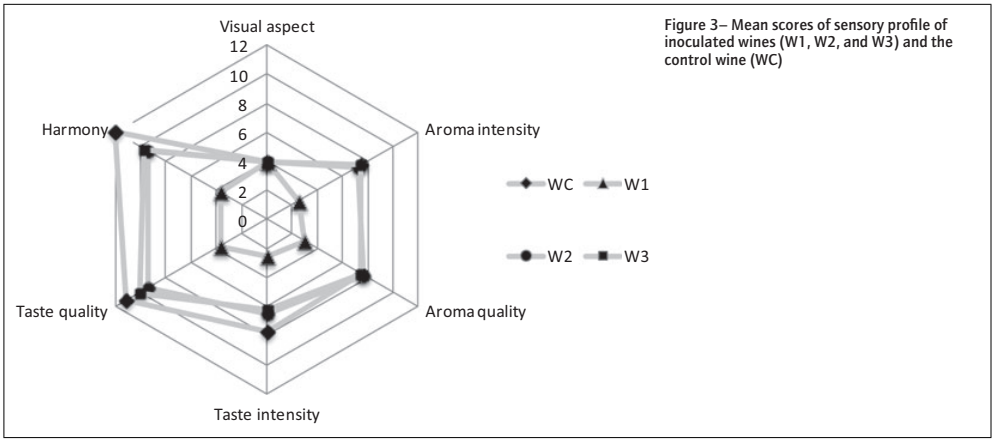

Figure 3– Mean scores of sensory profile of inoculated wines (W1, W2, and W3) and the control wine (WC)

β-damascenone), which have been described previously as distinctive compounds in these wines, because of their high concentration and their low perception thresholds (Vilanova and Sieiro 2006; Palomo and others 2007). These compounds are associated with fruity and herbaceous aromas related to the freshness of the wine (Escudero and others 2004), which favorably contributed to the results of the sensorial analysis, without undermining the contribution of other compounds in such a complex matrix as wine.

Conclusions

In summary, this work shows that W1 Albariño white wines have a higher concentration of mannoproteins and aromatic compounds (terpenes and norisoprenoids) than the other wines studied. The analysis of the retention capacity of the colloidal fraction of W1 for the main terpenes found (geraniol and linalool) suggests that mannoproteins are involved in this behavior. The sensory analysis carried out confirms that wine W1 is preferred by the tasters, so the selection of mannoprotein-overproducing wine yeasts could be an interesting strategy to use in the production process of Albariño white wines to obtain wines of better quality.

Acknowledgments

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CHAPTER 3

Effect of short ageing on lees on the mannoprotein content, aromatic profile and sensorial character of white wines.

Submitted

CAPÍTULO 3

EFECTO DE TIEMPOS CORTOS DE CRIANZA SOBRE LÍAS EN LA CONCENTRACIÓN DE MANOPROTEÍNAS, EL PERFIL AROMÁTICO Y EL CARÁCTER SENSORIAL DE LOS VINOS BLANCOS DE ALBARIÑO DE LA D.O. RÍAS BAIXAS

OBJETIVO

De los resultados obtenidos en el capítulo 2 de la presente memoria se evidencia que la mayor concentración de manoproteínas presente en la fracción coloidal de la cepa *S. cerevisiae* 1 favorece la retención de compuestos aromáticos y contribuye a la mejora de su calidad sensorial. Dado que la crianza sobre lías favorece, entre otros aspectos, la liberación de manoproteínas al vino, el principal objetivo del presente capítulo fue el de estudiar el efecto de la crianza sobre lías de *S. cerevisiae* 1 en la calidad de los vinos blancos de Albariño de la D.O. Rías Baixas.

PLAN DE TRABAJO

Para la realización del objetivo propuesto se llevaron a cabo las siguientes tareas:

1. Estudio en bodega a escala piloto (30 L) del efecto de la crianza sobre lías en la calidad de los vinos Albariño D.O. Rías Baixas elaborados con la cepa *S. cerevisiae* 1, determinando mediante análisis instrumental en muestras tomadas en distintos tiempos (fin de vinificación, 10, 20, 30, 40 y 50 días) los siguientes parámetros enológicos: grado alcohólico, acidez total, acidez volátil y pH.

2. Aislamiento, hidrólisis y cuantificación de la fracción de manoproteínas de los vinos correspondiente a cada uno de los tiempos de crianza

sobre lías.

Identificación y cuantificación de los compuestos volátiles de los vinos

3. Análisis sensorial de los vinos obtenidos y su relación con el tiempo de crianza sobre lías.

RESUMEN

En este trabajo se llevó a cabo un estudio del efecto de una crianza corta sobre lías en la calidad final de vinos blancos Albariño. Para ello, se obtuvieron vinos blancos Albariño fermentados con la cepa de levadura *S. cerevisiae* 1 que se mantuvieron en contacto con sus lías durante diferentes tiempos: 10, 20, 30, 40 y 50 días. Como control experimental se utilizó el vino sin envejecimiento sobre lías. En cada uno de los vinos obtenidos y para cada tiempo de envejecimiento se determinó el contenido en manoproteínas y el perfil de compuestos del aroma. Las manoproteínas aumentaron en el vino durante los primeros 20 días, conforme con las primeras etapas del proceso de autolisis de las levaduras. En tiempos posteriores de envejecimiento (40-50 días) se produjo una disminución de la fracción proteica y un incremento de la manosa polimérica en el vino, siendo este un comportamiento asociado a etapas tardías de la autolisis en las que se produce la liberación de péptidomananos al vino. El estudio de la fracción de aromas del vino reveló que en el caso de los aromas varietales (terpenos y norisoprenoides), los más característicos de este tipo de vinos, la concentración de la mayoría de los compuestos identificados fue mayor en los vinos con 20 días de envejecimiento sobre lías, lo que puede deberse tanto a la capacidad de absorción de las manoproteínas, demostrada en el capítulo anterior, como a la acción de β-glicosidasas liberadas durante la autolisis de las levaduras. La mayor parte de los esteres y acetatos, cuantitativamente el segundo grupo de compuestos aromáticos en estos vinos e involucrados en el frescor y aromas característicos de estos vinos, tuvieron un comportamiento similar, y su concentración fue mayor en los

vinos después de 20 días de envejecimiento sobre lías. Este comportamiento se ha asociado, entre otros factores, a la acción de esterasas liberadas al vino durante la autolisis. El análisis sensorial demostró la influencia del proceso de envejecimiento sobre lías en las características sensoriales de los vinos obtenidos. De los descriptores analizados, se observaron diferencias significativas en relación al aroma y sabor, mientras que el color fue similar en todos los vinos. Todos los vinos con envejecimiento sobre lías fueron mejor puntuados que el vino control. Los vinos envejecidos durante 20 días fueron los mejor evaluados por el panel de catadores coincidiendo con el hecho de que eran los vinos que presentaban un mayor contenido de compuestos varietales y otros compuestos del aroma, así como una mayor concentración de manoproteínas. Estos resultados indican que existe un tiempo óptimo de envejecimiento sobre lías para este tipo de vinos, y que una vez sobrepasado este, se afectan una serie de parámetros sensoriales que disminuyen su aceptabilidad.

Effect of short ageing on lees on the mannoprotein content, aromatic profile and sensorial character of white wines

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Abstract

In this work, a study was made of the effect of a short aging on lees during different times (10, 20, 30, 40 and 50 days) on the mannoprotein content, aromatic profile and sensorial character of white wines. The results obtained showed that the white wines with the best sensorial character were obtained after 20 days of aging on lees. This time was also related with the highest concentration of some key aroma compounds and mannoproteins. Further aging times decreased the sensorial quality of the wine, also modifying its analytical composition in both, aroma compounds and mannoproteins. Therefore, a short ageing on lees could be a successful post-fermentative alternative in Albariño white wines to enhance their typical aromatic and organoleptic characteristics and to produce more distinctive wines.

Keywords: white wines, yeast, lees, aging on lees, aroma compounds, mannoproteins.

1. Introduction

Ageing on lees is an oenological practice, which involves the contact of the wine obtained after alcoholic fermentation with the resting yeast. Lees are formed by microorganisms (mainly yeast), and by tartaric and inorganic matter (both in a minor proportion) (Perez-Serradilla & de Castro, 2008). Traditionally, only some white wines mainly from Burgundy and sparkling wines produced by the traditional method are left in contact with less (Loscos, Hernandez-Orte, Cacho, & Ferreira, 2009) but at present wine aging on lees is a more extended practice in many areas of wine production (Del Barrio-Galan, Perez-Magarino, Ortega-Heras, Wiliams, & Doco, 2011; Pati, Esti, Leoni, Liberatore, & La Notte, 2012; Rodrigues, Ricardo-Da-Silva, Lucas, & Laureano, 2012). Their main purpose is the improvement of the wine's sensorial character. The yeast autolysis process which takes place during wine ageing produces breakdown of cells membranes, release of intracellular components, liberation of hydrolytic enzymes, and hydrolysis of intracellular biopolymers into products of low molecular weights. Among the compounds released by yeast during aging on lees are the mannoproteins, consisting on small chains with one to four D-mannose residues which are linked to polypeptide chains on serine o threonine residues (Perez-Serradilla & de Castro, 2008). Mannoproteins like other breakdown products released to wine can modify significantly its sensorial properties (Pozo-Bayon, Martinez-Rodriguez, Pueyo, & Moreno-Arribas, 2009).

In young white wines the aroma is one of the principal quality criteria. These wines are characterized by a high intensity of fresh and fruity notes which depends on the contents of terpenes present in the grape, together with acetates and mono- and dicarboxylic acid ethylesters which appear during the fermentation process (Perez-Coello, Gonzalez-Vinas, Garcia-Romero, Diaz-

Maroto, & Cabezudo, 2003). A dual role has been attributed to yeast lees respect to its influence in wine aroma. They can contribute to the wine aroma balance, affecting positively the wine quality. But also the contact of wine with lees could decrease the content of volatile compounds, with a negative influence in the wine quality (Perez-Serradilla & de Castro, 2008). This behavior seems to be related with several variables, such as the characteristics of lees and the time that they are allowed to be in contact with the wine. For example, Loscos et al (2009) has found that lees from different yeast strains may have slightly different abilities to release volatile compounds derived from precursors. In the other hand, it has been observed that the contact of white wines with lees during 7 months modified their sensorial properties, decreasing the fruit and floral aromas (Bautista, Fernandez, & Falque, 2007). Using a short contact time with lees (20 days), the behavior observed was dependent of the grape variety. While in Airen wines most of the compounds increase its concentration, in Macabeo wines they decrease (Bueno, Peinado, Medina, & Moreno, 2006). The reported capacity of lees to interact with aroma compounds and potentially modify the sensory properties of the wine has also been related to mannoprotein fraction, considering that some of them can retain aroma compounds (Chalier, Angot, Delteil, Doco, & Gunata, 2007; Juega, Nunez, Carrascosa, & Martinez-Rodriguez, 2012)

Albariño grape is a typical variety from Galicia recognized by its high quality. White wines elaborated with Albariño grapes are most elaborated as young wines and they use to contain high concentrations of terpenes and are dominated by fruity and floral odors (Carrascosa, Bartolome, Robredo, Leon, Cebollero, Juega, et al., 2012; Vilanova, Genisheva, Masa, & Oliveira, 2010). Nowadays, there are not studies about the impact of short ageing on lees in the aroma composition and final quality of these wines, in the spite of this practice is used empirically by some producers with the purpose to obtain a distinctive character in the final wine. In the present work we have investigated the effect of different times in the aging on lees of Albariño white wines, evaluating its impact on the mannoprotein content, aroma profile and sensorial character.

2. Materials and methods

2.1- Must, yeast and fermentation conditions. The must used in this study was from *Vitis vinifera* cv. Albariño grapes (vintage 2009) and was supplied by the winery Terras Gauda, Galicia, Spain. The must was inoculated with *Saccharomyces cerevisiae* strain 1, a locally-selected yeast (Carrascosa, et al., 2012) and fermented in 30L stainless steel tanks. Fermentation experiments were carried out in triplicate. The temperature was set to 18ºC. Fermentation was followed by the sugar consumption, and the reducing sugar (Flanzy, 2003) during fermentation was determined until 40 days. The obtained wines were aged on its lees during different periods: 10 days (W10), 20 days (W20), 30 days (W30), 40 days (W40) and 50 days (W50). A control wine (CW) was prepared without aging on lees. Predominance of the selected yeast in the fermentation tanks was verified by studying the mitochondrial DNA profile at the end of the fermentation (Querol, Barrio, & Ramon, 1992). 1500 mL samples were taken from each tank and used in the experimental and sensory analysis. They were cleared by centrifugation at 1800 x g, 15 min. Conventional parameters in the wines (alcoholic grade, total acidity, volatile acidity, pH, tartaric and malic acid) were determined by the European Commission methods (EC, 1990) at the end of the fermentation and after 50 days of aging.

2.2- Precipitation, hidrolysis, and cuantification of mannoproteins. The procedure described by (Segarra, Lao, LopezTamames, & DeLaTorreBoronat, 1995) et al., 1995 was used for the isolation of colloidal fraction containing mannoproteins. 40 ml of ethanol (96% v/v) and 400 µl HCL (1N) were added to 8 ml of wine. After 18 h of incubation at 22 ºC, the tubes were centrifuged (1800 x g, 20 min), after which the supernatant was discarded and the pellet was washed three times in ethanol (96%, v/v). For the determination of the sugar composition of mannoproteins, the samples obtained were hydrolyzed at 100 ºC for 24 h in a

closed vial containing 1 ml of 2 M trifluoroacetic acid and 0.5 ml myo-inositol (0.1 % w/v, internal standard) solution. After hydrolysis, the mixture was evaporated to dryness under vacuum.

The dried hydrolyzed residue was silylated following the procedure described by (Nunez, Carrascosa, Gonzalez, Polo, & Martinez-Rodriguez, 2006). Briefly, the sample was dissolved in 100 ml of anhydrous pyridine, and 100 ml of trimethylsilylimidazole, 100 ml of trimethylclorosilane, 100 ml of n-hexane and 200 ml of deionized water were sequentially added, shaking during each step. Trimethyilsilyl derivatives (1µl) were analysed on a Hewlett-Packard 6890 Chromatograph (Palo Alto, CA, USA), equipped with a flame ionization detector (FID) and split/splitless injector. Samples were injected on a Carbowax 20M column (30 m X 0.25 mm) coated with a stationary phase of 0.25 mm thickness. Temperatures were as follows: injector and detector, 220 ºC; oven, held at 175 ºC for 15 min, then increasing 15 ºC/min to 200 ºC during 13 minutes and finally programmed at 30 ºC/min to 270 ºC during 20 minutes. The carrier gas was helium (10 psi, split 1/15). Response factors were calculated with a series of pure standards at different concentrations using myo-inositol as internal standard. The identification of the mannose present in the samples was carried out by comparing the retention time of the peaks with those of pure standard. Each sample was analyzed by triplicate. Results were expressed as mg/L of polymeric mannose in the wine. The concentration of protein moieties was determined following the Bradford method (Bradford, 1976), based in the reaction of the protein with the Coomasie blue G-250. Absorbance was determined at 595 nm 15 min. after the addition of the reactive. The results were expressed in mg of bovine seroalbumine (BSA)/L.

2.3- Volatile Compounds. The extraction of volatile compounds was automatically performed by using a CombiPal system (CTC Analytics AG, Zwingen, Switzerland) provided with a 50/30 µm Divinylbencene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fiber of 2 cm length (Supelco, Bellefonte, PA. USA). The extraction was performed in the

headspace of the vial for 20 minutes at 40 ºC. The desorption was performed in the injector of the GC chromatograph (Agilent 7890) in splitless mode for 12 minutes at 280 ºC. After each injection the fiber was cleaning for 30 minutes avoiding any memory effect. All the analyses were performed in triplicate.

An Agilent MSD ChemStation Software was used to control the gas chromatograph (Agilent 7890). For separation, a fused silica CP-WAX 57CB column (50m X 0,25mm X 0,39mm film thickness) from Varian (Houten, The Netherlands) was used. Helium was the carrier gas (1 mL/min). The oven temperature was programmed as follows: 60 ºC as initial temperature, held for 5 minutes, followed by a ramp of temperature at 2 ºC/min to 120 ºC and 3 ºC/min to 215 ºC, and then held for 25 minutes.

For the MS system (Agilent 5973N), the temperatures of the manifold and transfer line were 150 and 230 ºC respectively; electron impact mass spectra were recorded at 70 eV ionization voltages and the ionization current was 10 µA. The acquisitions were performed in selected-ion- monitoring (SIM) mode. The signal corresponding to a specific ion of quantification was calculated by the data system. Quantitative data were obtained by calculating the relative peak area (or TIC signal) in relation to that of the internal standard used for each compound. Calibration curves of each compound were performed using a model wine (4 g/L tartaric acid, 10 % v/v ethanol and pH=3) spiked with the commercial pure reference compounds at five different levels of concentration covering the concentration ranges expected in wines.

2.4- Sensory analysis. A panel of experts comprised of eight judges carried out sensory evaluation of the wines. The tasting card used was the official Rías Baixas index card (Carrascosa, et al., 2012). Wine samples were evaluated at 15º C. The scores used were penalizing scores so better quality wines receive a lower score. Six variables (visual examination, aroma intensity, aroma quality, taste intensity, taste quality and harmony) were proposed for assessment, and a scale of 7 categories designed (excellent: 0–7, very good: 8–23, good: 24–44, correct: 45–52, ordinary: 53–78, defective: 79–90, eliminated: >90). The mode of

the scores given by the eight tasters was used to arrive at the final score for each parameter corresponding to the sensorial characteristics of wine.

2.5- Statistical analysis. Significant differences among the data obtained from the volatile composition of the wines aged on lees during different periods were estimated by applying analysis of variance (ANOVA). The Tukey least significant differences (LSD) test was used to evaluate the significance of the analysis. The program used was SPSS 16.0 for Windows, version 16.0.1 (Nov. 2007).

3. Results and Discussion

The inoculated strain prevailed during the elaboration process, fermenting the must to dryness (1.2 ± 0.0 residual sugars). Table 1 shows the values for different chemical parameters of the wine at the end of the fermentation and after 50 days of aging on lees. As can be seen, no significant differences ($p<0.01$) were observed among them, demonstrating that these parameters were not affected by the aging on lees. In all cases, the values obtained are within the normal ranges found for these wines (Carrascosa, et al., 2012; Zamúz & Vilanova, 2006) and show that the vinification was satisfactory.

In the figure 1 is represented the changes in protein and polymeric mannose during the aging on lees. During the first 10 days there are slight decreases in the mannoprotein concentration, followed by an increase at 20 days of polymeric mannose, which suggest the beginning of the autolysis process. During the first stages of autolysis, the β-glucanases act on the yeast cell wall releasing mannoproteins covalently linked to the glucan in the cell wall (Pozo-Bayon, Martinez-Rodriguez, Pueyo, & Moreno-Arribas, 2009). Later on, the behaviour observed is also consistent with the yeast autolysis process. Protein moieties of mannoproteins are hydrolysed by proteases to low molecular peptides, while the β-glucanases degrades the glucans that are still linked to mannoproteins, releasing small peptidemannans in the wine (Rodrigues,

Ricardo-Da-Silva, Lucas, & Laureano, 2012), which are detected as a new increase in polymeric mannose.

In table 2 are presented the major volatile compounds identified in the wines aged on lees at different times. Higher alcohols, ranging from to 2.93 mg/L to 181.59 mg/L, were the most abundant compounds. In all cases, the concentration of the three identified compounds (3 methyl 1 butanol, 1 hexanol and 2 phenylethanol) was under 300 mg/L, which is the threshold at which alcohols can negatively affect the wine (Flanzy, 2003). 1 hexanol was not modified during aging on lees, while 3 methyl 1 butanol and 2 phenylethanol decreased during the first 20 days of aging, increasing at 30 days and decreasing again later. Some higher alcohols with high molecular weight, such as 2 phenylethanol, can be absorbed on the yeast cell wall and its concentration in the wine can be enhanced with the yeast cell wall lysis (Masino, Montevecchi, Arfelli, & Antonelli, 2008). In the present context, the increase of 2 phenylethanol and 3 methyl 1 butanol in the wine is in accordance with some analytical evidences of the yeast autolysis, suggesting that it can contribute to the release of these compounds from lees to the wine.

Esters and acetates were quantitatively the second group in volatile compounds. These compounds are responsible in part for the fresh and fruity aroma of young white wines (Antalick, Perello, & de Revel, 2010). A total of 7 of these compounds were identified in the wines tested: Isoamyl acetate, ethyl hexanoate, hexyl acetate, ethyl octanoate, ethyl decanoate, diethyl succinate, and 2 phenylethanol acetate. The highest concentration for most of the individual compounds was found in wines aging on lees after 20 days, decreasing later on. It has been described that the hydrolysis and esterification of esters can be strongly affected by activity of esterases, which are liberated after alcoholic fermentation and are associated to autolysis process (Bueno, Peinado, Medina, & Moreno, 2006). Esterase activity can be involved in the global decrease of these compounds after 20 days, coinciding with more advances stages of autolysis.

In the case of the terpenes and norisoprenoids identified (linalol, α-terpineol,

terpin-4-ol, β-damascenone, α-ionone and β-ionone) the highest concentration for most of them was found at 20 days of aging on lees, except for nerol and α −ionone, which concentration remains constant during aging on lees. The yeast strain used here for fermentation and aging on less (*Saccharomyces cerevisiae* strain1) can influence the volatile profile of white wines elaborated with Albariño must when they are used as single inoculum, increasing the final concentration of terpenes and norisoprenoids in the final wine (Carrascosa, et al., 2012). Apparently, some mannoproteins are related with this behaviour, at least for some compounds such as linalool, which can be absorbed for specific mannoproteins released by this strain (Juega, Nunez, Carrascosa, & Martinez-Rodriguez, 2012). Also, the β- glucosidases released during autolysis can contribute to the increase of these compounds in early stages of autolysis. These enzymes are able to break the glycoside bound of terpenes and norisprenoids, releasing the free aromatic form that consequently contribute to the characteristic aroma in wine (Liberatore, Pati, Del Nobile, & La Notte, 2010).

Butyrolactone was the only lactone identified in the wines and its concentration ranged between 4.16 mg/L and 6.51 mg/L. At these levels, lactones can contribute to the floral and fruity character of the wines (Perez-Serradilla & de Castro, 2008). Octanoic acid was the fatty acid identified, and it had the highest concentration in the wines of 20 days of aging on lees, also coinciding with the first stages of autolysis. In previous studies was demonstrated that the presence of lees can increase the concentration of fatty acids in wine, due to desorption phenomena occurred after fermentation and caused by yeast autolysis (Bautista, Fernandez, & Falque, 2007; Bueno, Peinado, Medina, & Moreno, 2006).

The results obtained from the sensorial analysis of the wines are represented in the figure 2. It can be observed that the aging time on lees can influence the sensorial evaluation of the wines. The scoring used on the card is based on a penalizing system, therefore the best wines are those with the lowest scores. Aging on less increased the acceptability of the wines, being the wines aged 20 days (W20) the best considered. The visual aspect was similar in all the wines, indicating that the differences observed were mainly related with

aroma and taste. The wines were sorted according their preference in the following way: W20>W30>W40>W50>W10>WC. This distribution indicates that between 10 and 20 days of aging on lees the wines acquire their better properties, decreasing it afterwards. These results agree with the analytical ones, and it can be observed that the chemical composition of the wines influences the behavior observed during the sensorial analysis. There was an optimum point for aging on lees related with the best sensorial quality of the wine. The best scored wines (W 20) were also the wines with the highest concentration of terpenes, norisoprenoids, esters and acetates, confirming that these compounds are significantly involved in the quality of the sensorial attributes of these kind of wines (Carrascosa, et al., 2012). Also, the preferred wines had the highest amount of mannoproteins, supporting its role at least in the putative retention of some aroma compounds (Juega, Nunez, Carrascosa, & Martinez-Rodriguez, 2012).

After 20 days of aging on lees, breakdown process associated to yeast autolysis modified the chemical composition of the wine, also transforming its sensorial properties. Anyway, any time of the aging process was favourable for the sensorial character of the wine, comparing with the results obtained for the control wine (WC). The autolysis process can contributes to modify positively the sensorial character of the wines trough the aging time on lees, but also it can negatively affect the sensorial properties of the white wines, mainly after several months of aging on lees (Bautista, Fernandez, & Falque, 2007).The optimum time for aging on lees will depends on several variables related with the winery process, but the yeast strain has a pivotal role and this point should be considered when this procedure is used (Bautista, Fernandez, & Falque, 2007; Carrascosa, et al., 2012).

In conclusion, the yeast used in this work to ferment and aging on less the white wines produces the wines with the best sensorial character after 20 days of aging on lees. This time is also related with the highest concentration of some key aroma compounds and mannoproteins. Further aging times decrease the sensorial quality of the wine, also modifying its analytical composition in both,

aroma compounds and mannoproteins. Although similar results were obtained in two different vintages (data not shown), the identification of an analytical marker capable to define an optimal aging time on lees could be interesting from the practical point of view, avoiding the putative interference of the multiple variables involved in the fermentation process.

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Article 3

Tables and Figures

Table 1. Chemical parameters in Albariño wines, at the end of the fermentation (CW), and after 50 days of contact with wine lees (W50).

Parameters	CW	W50
Ethanol (% v/v)	12.2 ± 0.06	12.3 ± 0.06
pH	3.4 ± 0.01	3.4 ± 0.02
Total acidity (g/L)	6.5 ± 0.00	6.5 ± 0.06
Volatile acidity (g/L)	0.2 ± 0.01	0.2 ± 0.01
A. Tartarico (g/L)	3.3 ± 0.12	3.3 ± 0.06
A. Malico (g/L)	3.3 ± 0.17	3.4 ± 0.15

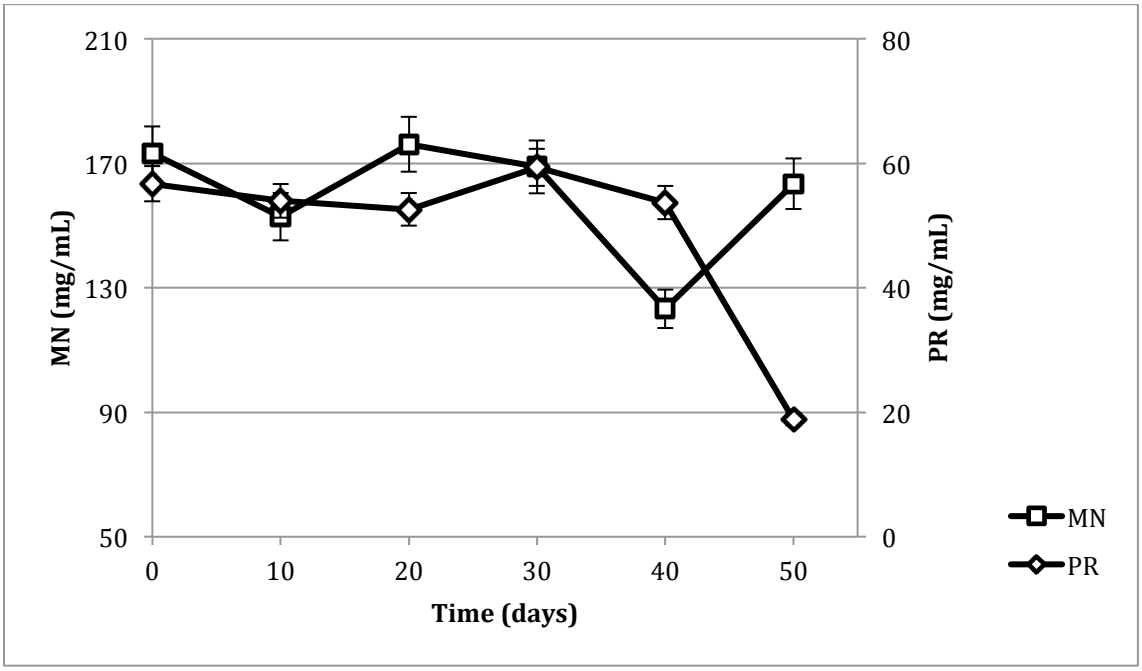

Figure 1. Concentration of proteins (PR) and polymeric mannose (MN) expressed in mg/L, in the wines aged on lees for different periods of time (0-50 days).

Table 2. Content of each aroma compounds identified in the wines in the control wine (CW) and in wines aged on lees at different times (10, 20, 30 40 and 50 days). Results are presented as mean ± SD

Concentration (mg/L)	CW	W10	W20	W30	W40	W50
Higher alcohols						
3 methyl 1 butanol	178.41a ± 5.92	152.5b ± 0.63	127.5c ± 2.47	181.59a ± 5.72	116.9c ± 10.85	161.05b ± 1.67
1 hexanol	3.33a ±0.11	3.06 a ±0.75	3.05a±0.06	3.93a±0.30	2.93a ±0.04	3.33a ±0.03
2 Phenylethanol	12.20a ±0.00	11.20a±0.02	10.9a±0.02a	14.6b±0.00	10.91a ±0.00	12.54a±0.00
Lactones						
Butyrolactone	5.70a±0.02	4.44bc±0.95	4.16b±0.01	6.51d±0.08	4.41bc ±0.01	5.19ac ±0.05
Esters and acetates						
Isoamyl acetate	2.03a ±0.15	1.8a±0.31	2.29a±0.12	2.05a±0.06	1.36b±0.06	2.05a±0.19
Ethyl hexanoate	0.80a±0.02	0.58b±0.09	0.86a±0,05	0.84±0.03a	0.56b±0.03	0.72a±0.00
Hexyl acetate	0.79a±0.00	0.70a±0.07	0.81a±0.03	0.74a±0.01	0.60b±0.02	0.84a±0.02
Ethyl octanoate	1.12a±0.01	0.57b±0.02	0.9c±0.01	1.03d±0.02	0.76e±0.00	0.64f±0.04
Ethyl decanoate	0.17a±0.00	0.08a±0.00	0.18a±0.00	0.16a±0.00	0.11a±0.00	0.18a±0.00
Diethyl succinate	3.65ab±0.10	3.84bd±0.00	4.86c±0.08	4.55c±0.07	4.16d±0.07	3.44a±0.09
2 phenylethanol acetate	0.22a±0.00	0.21a±0.02	0.24b±0.00	0.22a±0.00	0.18c±0.00	0.21a±0.00
Fatty acids						
Octanoic acid	2.27a±0.81	5.42b±0.36	6.76c±0.73	4.94b±0.38	4.67b±0.32	6.29c±0.26
Terpenes						
Linalool	0.04a±0,00a	0.04a±0.00	0.05b±0.00	0.04a±0.00	0.03a±0.00	0.04a±0.00
Terpinen-4-ol	0.0005a±0.00	0.03a±0.00	0.04b±0.00	0.03a±0.00	0.03a±0.00	0.03a±0.00
α-terpineol	0.02a±0.00	0.016a±0.00	0.021b±0.00	0.020a±0.00	0.015a±0.00	0.021b±0.00
Nerol	0.013a±0.00	0.009a±0.00	0.01a±0.00	0.009a±0.00	0.01a±0.00	0.009a±0.00
Eugenol	0.081a±0.00	0.083b±0.00	0.081a±0.00	0.082b±0.00	0.08a±0.00	0.083b±0.00
Norisoprenoids						
ß-Damascenone	0.002a±0,00a	0.001a±0.00	0.0031b±0.00	0.0025a±0.00	0.0025a ±0.00	0.0026a±0.00
α-ionone	0.013a±0.00	0.013a±0.00	0.013a±0.00	0.013a±0.00	0.013a±0.00	0.013a±0.00
ß-ionone	0.04a±0.00	0.04a±0.00	0.042b±0.00	0.040a±0.00	0.040a±0.00	0.041b±0.00

a, b, c,- Same letter in the same row indicates absence of significant differences ($p < 0.05$).

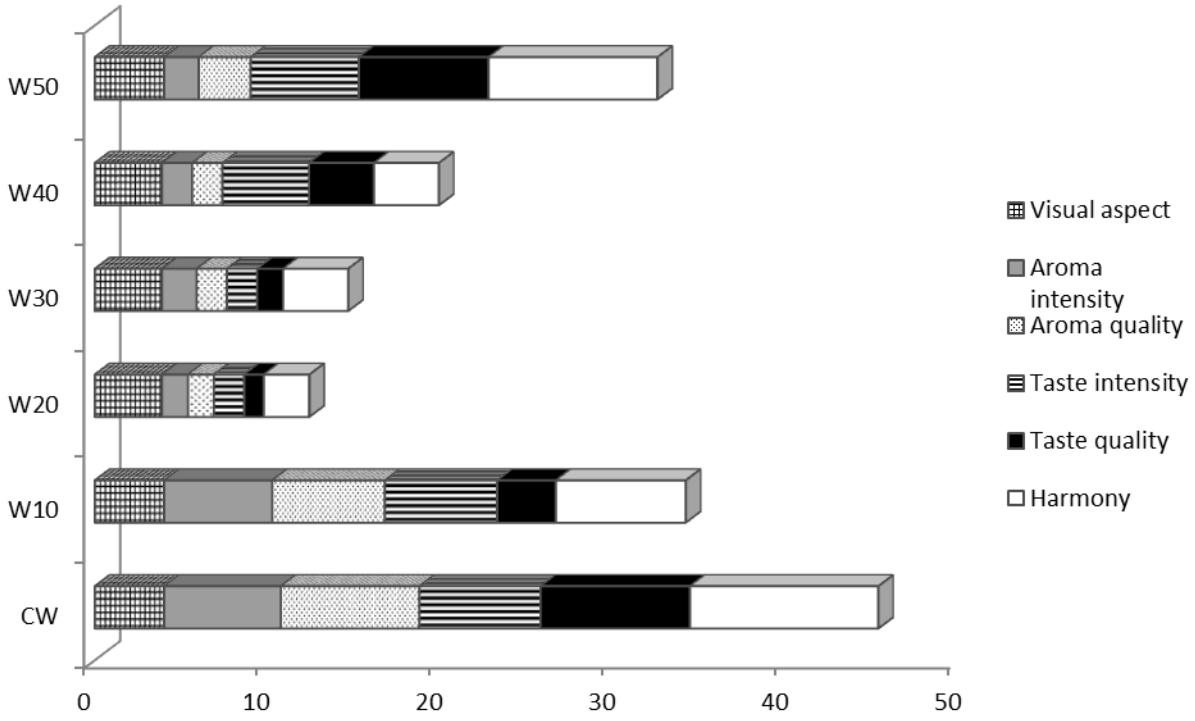

Figure 2. Mean scores of sensory profile of the wines aged on lees (W10, W20, W30, W40 and W50) and the control wine without aging (CW)

CHAPTER 4

Chemical evaluation of white wines elaborated with a recombinat *Saccharomyces cerevisiae* strain overproducing mannoproteins.

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CAPÍTULO 4

EVALUACIÓN QUÍMICA DE VINOS BLANCOS ALBARIÑO DE LA D.O. RÍAS BAIXAS ELABORADOS CON LA LEVADURA RECOMBINANTE SUPERPRODUCTORA DE MANOPROTEÍNAS *SACCHAROMYCES CEREVISIAE* EKD-13

OBJETIVO

A la vista de los resultados de los capítulos precedentes de la presente memoria, se evidencia que las manoproteínas pueden favorecer la retención de compuestos aromáticos que mejoran las características sensoriales de los vinos blancos obtenidos de *Vitis vinifera* cv. Albariño de la D.O. Rías Baixas. Por esta razón, el objetivo principal del presente capítulo fue estudiar el efecto de la vinificación de mosto de *Vitis vinifera* cv. Albariño con la cepa recombinante hiperproductora de manoproteínas *S. cerevisiae* EKD-13. Esta cepa se obtuvo en trabajos previos en el laboratorio de Microbiología del Instituto de Fermentaciones Industriales a partir de la cepa comercial *S. cerevisiae* EC1118.

PLAN DE TRABAJO

Para la realización del objetivo propuesto se llevaron a cabo las siguientes tareas:

1. Microvinificación *in vitro* (0,45 L) de mosto de *Vitis vinifera* cv. Albariño de la D.O. Rías Baixas con la cepa recombinante *S. cerevisiae* EKD-13 y su cepa parental *S. cerevisiae* EC1118, comprobando su predominancia mediante la técnica de tipado molecular del ADN mitocondrial.

2. Determinación de los siguientes parámetros enológicos: grado alcohólico, acidez total, acidez volátil, pH y azúcares residuales.

3. Aislamiento, hidrólisis y cuantificación de las manoproteínas totales.

4. Identificación y cuantificación de los compuestos volátiles.

5. Identificación y cuantificación de los parámetros de color y de los compuestos fenólicos de los vinos.

RESUMEN

En el presente trabajo, la cepa transgénica *S.cerevisiae* EKD-13 y su parental *S. cerevisiae* EC1118 se inocularon en mostos de *Vitis vinifera* var. Albariño, evaluándose su impacto en el perfil aromático y fenólico de estos vinos. Se utilizó como control un vino sin inocular. Se observó que la cepa transgénica *S.cerevisiae* EKD-13 se impuso a la microbiota espontánea presente en el mosto en una proporción del 90-100%, llevando a cabo la fermentación a sequedad y produciendo vinos típicos sin anomalías o alteraciones, demostrando su capacidad para vinificar mostos de Albariño. Los vinos obtenidos con la cepa transgénica *S.cerevisiae* EKD-13 presentaron una concentración media de manoproteínas de 593,5 mg/L, casi cuatro veces más que la concentración media descrita para este tipo de vinos (150 mg/L). El análisis de los principales compuestos volátiles presentes en los vinos demostró que el cambio más significativo observado en el vino fermentado con la cepa transgénica *S.cerevisiae* EKD-13 fue su elevada concentración 2-fenil etanol (139,4 mg/L), debido a la expresión en las condiciones de fermentación de uno de los marcadores de selección utilizados para construir la levadura recombinante. Sin embargo, no se observaron diferencias significativas en la concentración de terpenos y

norisoprenoides entre los distintos vinos, a pesar de la alta concentración de manoproteínas de los vinos transgénicos. De estos resultados se podría deducir que la capacidad de las manoproteínas para retener ciertos compuestos volátiles depende más de sus características que de la concentración de las mismas presentes en el vino. La influencia más significativa de la cepa EKD-13 en la composición fenólica fue la alta concentración de tirosol en los vinos elaborados con esta levadura, también debida a las modificaciones metabólicas derivadas de la construcción de la cepa transgénica, y que está relacionada con el alto índice de pardeamiento obtenido para estos vinos.

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Chemical evaluation of white wines elaborated with a recombinant *Saccharomyces cerevisiae* strain overproducing mannoproteins

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ABSTRACT

In this study, a recombinant *Saccharomyces cerevisiae* strain EKD13 overproducing mannoproteins has been used to obtain Albariño white wines. The inoculated strain prevailed and produced complete fermentation of the must, as also occurred in the case of spontaneous (non-inoculated) fermentation and in the must inoculated with the *S. cerevisiae* EC1118 strain. The analytical study of the wines obtained showed that the most important chemical differences among the wines produced with EKD-13, corresponded to the high concentration of mannoproteins, 2-phenyl ethanol and tyrosol. These differences were attributed to the expression, during must fermentation, of genes modified in the recombinant EKD-13 strain. The results obtained imply that this strain could be potentially useful to produce wines rich in mannoproteins that have distinctive characteristics compared to other similar wines, modifying the sensorial and technological parameters of the wines obtained.

1. Introduction

In the yeast cell wall two differentiated and intercommunicated regions can be identified. An internal one, composed of β-1,6 glucan (50–55% of dry weight of the wall) and chitin (1–2%), and an external one composed of β-1,6 glucan (3–14%) and mannoproteins (35–40%) (Klis, Mol, Hellingwerf, & Brul, 2002). Mannoproteins are glycoproteins comprised of 10–30% of protein and 70–90% of polysaccharides, of which approximately 98% correspond to mannose and 2% to glucose (Klis, Boorsma, & De Groot, 2006).

The use of mannoproteins from the cell wall of *Saccharomyces cerevisiae* (*S. cerevisiae*) has become increasingly important in winemaking in recent years. This is due to the different enological properties of mannoproteins, which allows the improvement of technological processes and/or sensorial characteristics of the wines. These include the capacity to influence the colloidal stability of a wine by protecting against protein haze and tartaric precipitation (Dupin et al., 2000; Monie-Ledoux & Dubourdieu, 1998), and positive sensorial effects based on astringency reduction and colour stabilization in red wines (Escot, Feuillat, Dulau, & Charpentier, 2001; Fuster & Escot 2002; Vidal et al., 2004), foam stabilization in sparkling wines (Nunez, Carrascosa, Gonzalez, Polo, & Martinez-Rodriguez, 2006), and favourable retention of some aromatic compounds (Chalier, Angot, Delteil, Doco, & Gunata, 2007; Juega, Nunez, Carrascosa, & Martinez-Rodriguez, 2012).

All these properties are attractive for the manufacturers, who have found that wines enriched with mannoproteins have an added value as they help to improve the technological and sensorial characteristic of the wine.

In wine production, enrichment in mannoproteins mainly takes place during alcoholic fermentation and after yeast autolysis, by the action of exogenous β-glucanase enzymes in the yeast cell wall (Feuillat, 2003). In most cases, both processes only increase the mannoprotein content of the wines by a few mg/l, so several strategies have been described that attempt to increase the mannoprotein concentration in wine. These include the selection of yeasts (Carrascosa et al., 2012), the addition of mannoprotein-rich extracts (Guadalupe, Martinez, & Ayestaran, 2010), and the obtention of yeast overproducing mannoprotein, either using classical genetic protocols (Gonzalez-Ramos & Gonzalez, 2006) or genetic engineering procedures (Gonzalez-Ramos, Cebollero, & Gonzalez, 2008). Using this last strategy, the transgenic yeast *S. cerevisiae* EKD-13 was constructed, and has been shown to release increased amounts of mannoproteins after fermentation of a Sauvignon Blanc must (Gonzalez-Ramos et al., 2008.

Wines of the Albariño grape are considered to be among the highest quality young white wines in Spain. Wines obtained from this grape variety are characterised by their intense floral and fruity aromas (Vilanova & Siero, 2006; Zamuz & Vilanova, 2006). Terpenes, owing to their high concentration and low sensorial threshold, are considered to be among the main components responsible for the characteristic aroma of these wines (Carballeira, Cortés, Gil, & Fernández, 2001; Falqué, Fernández, & Dubourdieu,

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2001). Previous studies have shown that the inoculation of strains of *S. cerevisiae* selected for fermentation produces changes in the aromatic profile and phenolic composition of the wines obtained, improving their sensorial characteristics (Carrascosa et al., 2012). It has also been found that yeasts that release larger amounts of mannoproteins produce better quality wines (Juega et al., 2012). The main objective of this work is, therefore, to determine the influence of inoculation of Albariño must with the recombinant strain *S. cerevisiae* EKD-13 overproducing mannoproteins, assessing its impact on the aromatic and phenolic composition of the wines obtained.

2. Materials and methods

2.1. Yeast strains, media and culture conditions

The Albariño must was inoculated with the following strains of *S. cerevisiae*: EC1118, (Lallemand Inc., Montreal, Canada), and the recombinant strain EKD-13, which was obtained from EC1118 by Gonzalez-Ramos et al. (2008). Briefly, the recombinant strain EKD-13 was obtained by deleting two copies of the *KNR4* gene, which were replaced by two alleles that were the sizes expected for *ARO4-OFP* and *KanMX4* replacement, conferring resistance to *p*-fluoro-DL-phenylalanine (PFP) in presence of tyrosine and geneticine, respectively. Deletion cassettes were prepared consisting of the marker gene flanked by 500 base pairs corresponding to promoter and terminator regions KNR4, respectively. The different components of the cassettes were cloned on a plasmid using the technique of "first extension". Transformation of the EC1118 *S. cerevisiae* strain was carried out by the lithium acetate method described by Ito, Fukada, Murata, and Kimura (1983), as modified by Gietz and Woods (2002). The yeast strains were transformed with 20 μl of the appropriate PCR amplification reaction mixture. The resistance phenotypes of transformants were confirmed by replica plating on selection media with PFP in the presence of tyrosine and geneticin. Several transformants were analysed by extracting and amplifying the genomic DNA by PCR KNR4 locus.

To prepare the cultures, the selected strains were inoculated in 30 ml of YPD medium (2% glucose, 2% peptone, 1% yeast extract), and were incubated at 30 °C, 5000*g* for 24 h.

2.2. Microvinification assays

For vinification assays, yeast cell were washed twice with 0.9% NaCl saline solution and added to 450 ml of Albariño must (2010 vintage). The final concentration was set at 10^6 cfu/ml. As well as the fermentations inoculated with the selected strains (EC1118 and EKD-13), a non-inoculated (spontaneous) fermentation was carried out of the Albariño must. A total of 6 microvinifications were prepared for each of the selected strains, and the control. For the fermentation process, all wines analysed ($n = 18$) were maintained at 17 °C for approximately 30 days in a temperature-controlled system (Medilow, J.P Selecta, Barcelona, Spain). Fermentations were considered to be complete when the residual sugar concentration had dropped to lower than 5 g/l. At the end of fermentation, the samples were centrifuged at 10,000*g* for 15 min and the supernatant was conserved to be used for the remaining analytical determinations.

Follow up of the prevailing inoculated strains was monitored by studying mitochondrial DNA restriction profiles (Querol, Barrio, Huerta, & Ramón, 1992) for the non-inoculated wines and for the wines inoculated with EC1118, and by the method described by Gonzalez-Ramos et al. (2008) in the case of the recombinant yeast EKD-13, which implies phenotypic monitoring using geneticin as the selection marker. Samples were taken in different stages of the fermentation: initial phase (P1, 3 days); middle fermentation (P2, 15 days); and at the end of the fermentation (P3; 25 days). For each experimental sampling (P1, P2 and P3), serial decimal dilutions were prepared in saline solution (0.9% NaCl) and plated (20 ml) onto fresh YPD agar and incubated at 30 °C. The number of colony forming units (cfu) was assessed after 48 h of incubation. Results were expressed as cfu/ml. 50 isolated colonies were taken in each case to analyse the imposition rate. The imposition rate was determined by studying the mitochondrial DNA profile (non-inoculated and EC 1118 fermentation) and by phenotypic monitoring using geneticin as the selection marker (EKD-13). The imposition rate was expressed as a percentage. The basic parameters studied (total acidity, volatile acidity and pH) were determined by the European Commission methods (Europeon community, 1990). Ethanol and glycerol levels were determined by the HPLC methodology described by Gonzalez-Ramos et al. (2008).

2.3. Isolation, hydrolysis and quantification of total mannoproteins

The procedure described by Segarra, Lao, Lopez-Tamames, and de la Torre-Boronat (1995) was used for the isolation of polysaccharides. 5 ml of ethanol (96% v/v) and 50 μl HCl (1 N) were added to 1 ml of wine. After 18 h of incubation at 22 °C, the tubes were centrifuged (1800*g*, 20 min), after which the supernatant was discarded and the pellet was washed three times in ethanol (96%, v/v). Samples obtained were hydrolyzed at 100 °C for 48 h in a closed vial containing 1 ml of 30% formic acid and 0.5 ml mio-inositol (0.1% w/v, internal standard) solution. After hydrolysis, the mixture was evaporated to dryness under vacuum.

The dried hydrolyzed residue was silylated following the procedure described by Nunez et al. (2006). Briefly, the sample was dissolved in 100 L of anhydrous pyridine, and 100 μl of trimethylsilylimidazole, 100 μl of trimethylclorosilane, 100 μl of n- hexane and 200 μl of deionized water were sequentially added, shaking during each step. The silylated derivatives present in the organic phase were immediately injected into the GC. Trimethyil-silyl derivatives were analysed on a Hewlett–Packard 6890 Chromatograph, equipped with a flame ionisation detector (FID) and split/splitless injector. Samples were injected on a Q1-30-25F column (30 m × 0.25 mm) coated with a stationary phase of 0.25 μm thickness. Temperatures were as follows: injector and detector, 220 °C; oven, held at 40 °C for 10 min, then increasing 7 °C/min to 150 °C, and finally programmed at 30 °C/min to 210 °C. The carrier gas was helium (12.5 psi, split 1/15). Response factor was calculated with mannose (Sigma Aldrich, MO, USA) at different concentrations using mio-inositol as internal standard. Identification of the mannose in the samples was carried out by comparing the retention time of the peak obtained with those of pure standard. Quantification was performed by comparing the relative areas obtained for the standard of each compound, with that obtained by the response factor of each standard substance, in the same conditions as the study sample.

2.4. Volatile analysis

Analysis of the major volatile compounds (higher alcohols) was performed by direct injection in a Hewlett–Packard 5890 series II gas chromatograph equipped with flame ionisation detection (FID) and a split/splitless injector. Separations were carried out on a Chrompack CP-WAX 57 CB fused silica capillary column (50 m × 0.25 mm i.d.) coated with a 0.25 μm thick polyethylene glycol stationary phase (Varian, Houten, The Netherlands) and the injector and detector temperatures were 220 °C. The temperature program was as follows: initial temperature 40 °C (10 min hold) and ramps of 5 °C/min to 200 °C and 20 °C/min to 210 °C during 20 min The carrier gas was helium (15 psi). A total of 50 ml of

3-pentanol (6 mg/ml 10% ethanol) was added as internal standard to 10 ml of wine, and 2 ml of wine with the internal standard was injected in the split mode. A ChemStation data system (HP 3365 series II, v. A.03.21) was used for data acquisition and processing. The compounds determined by this method were methanol, 2-phenylethanol, 1-propanol, 2-methyl-1-propanol, cis-3-hexanol, 1 hexanol, ethyl acetate and 2- and 3-methyl-1-butanol.

Minor volatile compounds (esters and acetates) were analysed by gas chromatography of the headspace extract obtained with a 100 mm poly-dimethylsiloxane coated fused silica fibre (Supelco, Bellefonte, PA, USA), in the conditions described by Pozo-Bayon, Pueyo, Martin-Alvarez, and Polo (2001), using methyl nonanoate as internal standard. The compounds determined by this method were phenyl- ethyl acetate, ethyl butyrate, ethyl hexanoate, hexyl acetate, butyl acetate, isopentyl acetate, isoamyl acetate, ethyl heptanoate, and diethyl succinate. The temperature of both injector and detector was 250 °C. The temperature program was as follows: initial temperature 70 °C and ramps of 5 °C/min to 200 °C and 3 °C/min to 215 °C. The carrier gas was helium (15 psi).

Free terpenes and norisoprenoids were fractionated by selective retention on SepPak Vac C-18, according to the procedure described by Vilanova and Sieiro (2006). The free fraction was eluted with pentane dichloromethane (2:1, 10 ml) and the eluate was dried over anhydrous sodium sulphate and concentrated to 0.5 ml, by evaporation with a stream of nitrogen, before GC analysis. Conditions used for chromatographic analysis were: injector temperature (250 °C), detector temperature (260 °C), injection type (splitless, 30 s) and injection size (1 μl). The temperature program was as follows: initial temperature 70 °C (7 min hold) and ramps of 2 °C/min to 120 °C and 3 °C/min to 215 °C during 25 min. The carrier gas was helium (14.5 psi). The compounds determined by this methodology were geraniol, linalool, nerol, terpin-4-ol, α-terpineol, b-citronerol, b-ionone, α-ionone and b-damascenone, using 3-octanol as internal standard.

In all cases, peak identities were assigned by comparing the relative retention times of the internal standard, with those of the standards of analytical quality, of over 99% purity, from Sigma–Aldrich. For quantification purposes, the relative area was obtained as the chromatographic peak area of each aroma compound divided by the area of the internal standard. Calibration curves in synthetic wines with each of the reference compounds (5 levels of concentration covering the concentration ranges expected in wines ×3 repetitions) were used, after checking the absence of significant matrix effects for most of the volatile analysed by the comparison of the slopes of the regression curves obtained in the synthetic and real wines.

2.5. Phenolic determinations and colour parameters

Total polyphenol index: The absorbance at 280 nm was directly measured in the wine using a 1-mm cell. The value of the total polyphenol index (TPI) was calculated by multiplying the absorbance ×10.

Total polyphenols and catechins: Wines were assayed for total polyphenols (TP) using the Folin–Ciocalteu reagent (Singleton & Rossi, 1965). Results were expressed as mg of gallic acid per litre. Also, they were assayed for catechins following the method of Swain and Hillis (1959). Results were expressed as mg of (+)-catechin per litre. In both cases, analysis was carried out in duplicate.

Colour measurement and test for browning potential: Wine colour intensity was determined by measuring absorbance at 420 nm (10 mm cell) using a Beckman Coulter DU-800 spectrophotometer (Fullerton, CA, USA). The wine browning potential was determined following the method of Cosme, Ricardo-da-Silva, and Laureano (2008). Test tubes were filled to 75% with the wine to be tested. Controls were sparged thoroughly with nitrogen and test samples were sparged with oxygen. All tubes were sealed and maintained at 55 °C for 5 days. After this time, the absorbance at 420 nm was measured in the wines using a 10 mm cell. The browning potential of a wine was calculated as the difference in absorbance between the oxygen-sparged wine and the control wine. Tests for browning potential were carried out in duplicate.

Analysis of phenolic compounds by liquid chromatography (LC): A Waters (Milford, MA, USA) liquid chromatography system equipped with a 2695 Alliance separation module, a 2996 photodiode-array detector (DAD), and a 2475 fluorescence detector was used. Separation was performed on a reversed-phase Waters Nova-Pak C18 (250 mm × 4.6 mm, 4 μm) column at room temperature. A gradient consisting of solvent A (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic acid, 78:20:2, v/v/v) was applied as follows: from 0 to 55 min, 100–20% A, 0–80% B, 1 ml/min; from 55 to 65 min, 20–0% A, 80–0% B, 0–100% methanol, 1–1.2 ml/min, from 65 to 75 min, 100% methanol, 1.2 ml; and re-equilibration of the column from 75 to 95 min. The detection conditions were: 210–360 nm (DAD); 280 nm and 310 nm for the emission and excitation filters, respectively (fluorescence detector). Identification of chromatographic peaks was achieved by comparing with retention times and UV spectra of phenolic standards. Quantification was carried out by external standard calibration curves. Gallic acid was quantified at 280 nm; hydroxycinnamic acids and their derivatives at 340 nm; and flavan-3-ols and tyrosol by their fluorescence response. Due to the lack of commercial standards, hydroxycinnamic derivatives were quantified using the free acid calibration curve, and procyanidin dimers were quantified using the (+)-catechin calibration curve.

2.6. Statistical analysis

Unless otherwise stated, data are the mean ± standard deviation from three independent experiments performed in duplicate. Significant differences among the data obtained from the volatile and phenolic composition of the wines elaborated with different yeasts were estimated by applying analysis of variance (ANOVA). This analysis was performed using the PC software package Statgraphics (version 2.1; Statistical Graphics Corp. Rockville, MD, USA).

3. Results

3.1. Fermentation of Albariño must using a S. cerevisiae transgenic yeast

As can be observed from Table 1, the two fermentations inoculated with the selected yeasts prevailed from the start. In the analysis of the non-inoculated fermentation, a very heterogeneous microbiota was identified, although since the middle phase of the fermentation process (phase P2) an unknown strain imposes and it was later the main responsible strain in the fermentation. Analysis of the viable yeast population in each of the fermentation steps showed that in the initial phase the population was similar for all the wines, but as fermentation progressed different behaviours were observed. During the middle phase of the fermentation the wine made with the EC1118 strain was found to present the greatest concentration of viable yeasts (5.2×10^7 cfu/ml), while the wine fermented with yeast EKD-13 presented the lowest concentration (2.6×10^6 cfu/ml). In the final phase of the fermentation this tendency was confirmed and there was a significantly lower concentration of viable yeasts in the wines fermented with the strain EKD-13 (3.8×10^4 cfu/ml) than in the other wines. There were no significant differences among the wines ($p < 0.05$) for the chemical parameters determined. The average values were as

Table 1
Imposition rate (IR) and viable concentration (VC) of inoculated and non-inoculated fermentations during three different sampling points: P1 (initial phase, 3 days), P2 (middle phase, 15 days) and P3 (end of the fermentation, 25 days). (mean ± SD, $n = 3$).

	P1		P2		P3	
	IR (%)	VC (cfu/mL)	IR (%)	VC (cfu/mL)	IR (%)	VC (cfu/mL)
Non-inoculated fermentation	Mixed profiles[a]	$2.7 \times 10^4 \pm 0.47$	81.5	$2.9 \times 10^7 \pm 0.99$	85.72	$7.7 \times 106 \pm 2.55$
Fermentation inoculated with strain EC1118	100	$3.1 \times 10^4 \pm 2.76$	99.7	$5.2 \times 10^7 \pm 2.14$	96.3	$8.2 \times 10^5 \pm 2.49$
Fermentation inoculated with strain EKD-13	98.98	$9.4 \times 10^4 \pm 0.12$	100	$2.6 \times 10^6 \pm 0.46$	100	$3.8 \times 10^4 \pm 1.75$

[a] At least 2 different yeast profiles were detected in the initial phase of the fermentation.

follows: alcoholic grade 11.9%, total acidity 6.6 g of tartaric acid/L, volatile acidity 0.2 g of acetic acid/L and pH 3.2. Although all wines ferment to dryness (<5 g/l of sugars) at the end of the fermentation process, the intake of glucose during the first stages of the fermentation process was significantly higher in inoculated wines, revealing the importance of using starter cultures in the must to accelerate the start of the fermentation process, which is when the must is most unstable, due to the high sugar concentration. Moreover, a significantly smaller quantity of glycerol was produced in the spontaneous fermentation, with values of around 2.6 g/l, compared to the 7.1 g/l and 7.4 g/l obtained with the fermentations inoculated with the EC1118 and EKD-13 strains, respectively. This could be interesting from the practical point of view, because a high content of glycerol could influence the final sensorial properties of the wine. In previous works, other authors have associated the use of selected yeast strains to carry out fermentation with an increased glycerol concentration in the final wine (Radler & Schutz, 1982; Grazia, Iorizzo, Vendetti, & Sorrentino, 1995).

3.2. Mannoproteins content

Fig. 1 shows the amounts of protein colloids and polymeric mannose determined in each of the final wines. The mean concentration of protein colloids present in the wines made with yeast strain EKD-13 was around 51 ± 2.4 mg/l, and was significantly higher ($p < 0.05$) than the concentration present when the EC1118 strain was used and in the control wine (30 and 22 mg/l, respectively). On the other hand, the mean polymeric mannose content in wines elaborated with transgenic yeast was 593.5 ± 22.9, around 40% more than in the wine produced with the EC1118 strain, which occupied second place.

3.3. Volatile composition of the wines

The analysis of the volatile fraction present in the wines identified a total of 27 aromatic compounds that are recorded in Table 2,

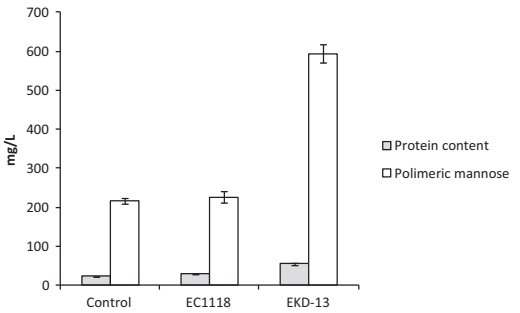

Fig. 1. Concentration of protein colloids and polymeric mannose (mg/l) in the different wines. The results represented are the mean ± standard deviation ($n = 3$).

classified into different chemical families. A total of 7 different alcohols were identified, with concentrations ranging from 300 mg/l in wines obtained from the non-inoculated fermentation of the Albariño must to 460.2 mg/l in wines produced with the EKD-13 yeast. This difference was mainly due to the concentration of the 2-phenyl ethanol (139.4 mg/l) present in the wine produced with the EKD-13 strain, which was around 5 times greater than that recorded in the wine made with the EC1118 strain, which had the second highest concentration. There was more than twice the concentration of 1-propanol (91.3 mg/l) in the wine made with the EKD-13 strain than in the other wines, and this value was higher than values usually recorded for these types of young white wines (20–40 mg/l) (Losada et al., 2011; Pérez-Coello, González-Viñas, García-Romero, Díaz-Maroto, & Cabezudo, 2003). In similar wines, 1-propanol has been associated with sensorial descriptors such as ripe fruit and alcohol (Peinado, Moreno, Bueno, Moreno, & Mauricio, 2004).

In these wines, 10 different acetates and ethyl esters were quantified and significant differences were found among the wines ($p < 0.05$), with the highest values usually associated with wines produced with the EC1118 strain. Isoamyl acetate was the major acetate, and no significant differences were observed among strains. Ethyl heptanoate was the majority ester, reaching a concentration of 120.55 mg/l in wines fermented with yeast EC1118. At concentrations lower than 100 mg/l, esters can positively influence the final aroma of a wine, giving it a fruity and floral character. However, if present in higher concentrations they tend to be associated with negative descriptors such as varnish or turpentine (Sumby, Grbin, & Jiranek, 2010). Taking this into account, the large amounts of ethyl heptanoate found in wines made with the EC1118 yeast could negatively affect sensorial perception.

Octanoic acid was the only organic acid identified and quantified and was only detected in wines fermented with yeast strain EKD-13. The concentration of octanoic acid found in wines made with the EKD-13 strain was above the perception threshold (0.5 mg/l) (Ferreira, Lopez, & Aznar, 2002), and could contribute to the freshness and balance of the fruity aromas present in the wine (Etievant, 1991).A total of 9 varietal aroma compounds were identified and quantified, of which 6 corresponded to non-glycosylated terpenes and 3 norisoprenoids. All three wines had a similar profile of varietal compounds, with the exception of geraniol, which was not found in wines made with the EKD-13 strain. In all cases, the concentrations of these compounds were similar to those recorded previously by other authors in similar wines (Rodríguez-Bencomo, Cabrera-Valido, Pérez-Trujillo, & Cacho, 2011; Vilanova, Zlatina, Masa, & Oliveira, 2010). Linalol was the most abundant terpene in all three wines, and was present at concentrations above the perception threshold (0.050 mg/l) (Etievant, 1991). However, significant differences were not observed ($p < 0.05$) in the amounts of linalol detected in the different wines. The norisoprenoids identified were β-damascenone and α and β-ionone, and significant differences among the wines were only detected in the case of β-damascenone, with higher values of this compound recorded in the non-inoculated wines (0.017 mg/l). This has been associated with floral and fruity flavours (Ribereau-Gayon,

Table 2
Concentration (mean ± SD, $n = 3$) of the aroma compounds: alcohols, acetates, ethyl esters, fatty acids, terpenes and norisoprenoids in the different wines. Results are expressed in mg/l.

	Non-inoculated fermentation	EC1118	EKD-13
Alcohols			
Methanol	56.68[a] ± 5.57	77.83[a] ± 7.21	47.15[a] ± 2.83
1 Propanol	36.9[b] ± 4.07	32.32[b] ± 1.92	91.34[a] ± 0.88
2 Methyl butanol	18.73[a] ± 0.80	19.7[a] ± 0.87	21.15[a] ± 0.07
3 Methyl butanol	144.33[a] ± 6.66	153.4[a] ± 5.83	134.82[b] ± 0.54
Cis-3-hexanol	18.45[b] ± 2.4	25.45[a] ± 3.77	20.88[b] ± 0.73
2 Phenyl ethanol	19.57[c] ± 2.29	26.89[b] ± 5.01	139.39[a] ± 7.26
1 Hexanol	5.66[b] ± 0.20	6.27[a] ± 0.08	5.47[c] ± 0.09
Acetates			
Isoamyl acetate	112.07[a] ± 5.10	110[a] ± 2.73	106.57[a] ± 0,37
Ethyl acetate	33.96[a] ± 9.59	22.44[b] ± 1.71	23.87[c] ± 1.36
Buthyl acetate	5.34[b] ± 0.18	5.03[b] ± 0.23	5.71[a] ± 0.19
Phenyl-ethyl acetate	5.63[b] ± 0.008	5.96[a] ± 0.15	6.1[a] ± 0.09
Hexyl acetate	n.d.	1.48[a] ± 0.02	1.47[a] ± 0.03
Isopentyl acetate	1.53[b] ± 0.016	5.8[a] ± 0.29	5.84[a] ± 0.66
Esters			
Ethyl heptanoate	73.05[c] ± 6.57	120.55[a] ± 10.52	88.68[b] ± 7.87
Ehtyl butyrate	3.29[c] ± 0.11	8.29[a] ± 0.67	4.17[b] ± 0.23
Ethyl hexanoate	1.34[c] ± 0.016	1.85[a] ± 0.06	1.5[b] ± 0.03
Diethyl succinate	n.d.	5.96[a] ± 0.15	6.1[a] ± 0.10
Fatty acids			
Octanoic acid	n.d.	n.d.	7.03 ± 0.40
Terpenes			
Linalool	0.098[a] ± 0.00	0.088[a] ± 0.00	0.085[a] ± 0.00
Terpin-4-ol	0.005[a] ± 0.00	0.003[b] ± 0.00	0.002[c] ± 0.00
α-Terpineol	0.0045[c] ± 0.00	0.005[b] ± 000	0.006[a] ± 0.00
β-Citronerol	0.025[a] ± 0.00	0.036[a] ± 0.00	0.049[a] ± 0.00
Nerol	0.001[a] ± 0.00	0.001[a] ± 0.00	0.0008[b] ± 0.00
Geraniol	0.036[a] ± 0.00	0.031[b] ± 0.00	n.d.
Norisoprenoids			
β-Damascenona	0.017[a] ± 0.00	0.015[b] ± 0.00	0.013[b] ± 0.00
α-Ionone	0.00059[a] ± 0.00	0.00058[a] ± 0.00	0.00053[a] ± 0.00
β-Ionone	0.000090[a] ± 0.00	0.000091[a] ± 0.00	0.000097[a] ± 0.00

n.d.: Not determined.
[a,b,c] Same letter in the same row indicates absence of significant differences ($p < 0.05$).

Glories, Maujean, & Dubordieu, 2003) and has a low perception threshold (0.5 μg/l) (Zhu-mei, Tao, Zhang, & Li, 2011).

3.4. Phenolic composition and colour of the wines

The total phenolic content, determined either as the absorbance at 280 nm (Total polyphenol index, TPI) or as the Folin–Ciocalteu reaction (Total polyphenols) was significantly higher for the wines elaborated with the recombinant strain EKD-13 in comparison to the wines fermented spontaneously (1.2 and 1.3 times higher for TPI and Total Polyphenols, respectively) and the wines inoculated with the commercial strain EC1118 (1.3 and 1.4 times higher for TPI and total polyphenols, respectively) (Table 3). In any case, these data were in the range of values for phenolic content of Albariño wines (Carrascosa et al., 2012). Concerning the colorimetric determination of catechins, the wines fermented spontaneously showed significantly higher values (54.2 mg/l, as mean) that the wines inoculated either with the EC1118 (33.5 mg/l) or the EKD-13 (32.9 mg/l) strains (Table 3). This same behaviour was observed for the colour intensity measured as absorbance at 420 nm (Table 3). However, the browning potential, which is an indicator of wine oxidability and is measured as absorbance at 420 nm after an accelerated oxidation process, was significantly higher for the wines fermented with the recombinant strain EKD-13 (Table 3), indicating that this wine would be less stable to oxidation.

Main individual phenolic compound identified in the LC chromatograms of wines were of different chemical structure: hydroxybenzoic acids (gallic acid), hydroxycinnamic acids and derivatives (caffeic acid and its derivative caftaric acid, *p*-coumaric acid and its derivative cutaric acid, and ferulic acid), phenolic alcohols and other related compounds (tyrosol and tryptophol), and flavan-3-ols [(+)-catechin, (−)-epicatechin and procyanidin dimers (B3, B1, B2 and B7) and trimers (C1)] (Table 4). Occurrence of most of these compounds were reported in previous studies on Albariño

Table 3
Color, total phenolic index (TPI), total polyphenols, total catechins and browning potential (BP) of the different wines (non-inoculated fermentation, wines elaborated using EC1118 strain and wines elaborated using EKD-13 recombinant strain). Data are the mean ± SD ($n = 2$).

	Non-inoculated fermentation	EC1118	EKD-13
Color (Abs 420 nm)	0.138[a] ± 0.008	0.115[b] ± 0.005	0.118[b] ± 0.004
TPI (Abs 280 nm)	9.41[b] ± 0.32	9.02[c] ± 0.20	11.5[a] ± 0.30
Total polyphenols (mg gallic acid/L)	277[b] ± 16	258[c] ± 9	345[a] ± 4
Total catechins (mg(+)- catechins/L)	54.2[a] ± 4.9	33.5[b] ± 1.6	32.9[b] ± 1.6
BP (Abs 420 nm)	0.0285[b] ± 0.0116	0.0332[b] ± 0.0105	0.0794[a] ± 0.0131

[a,b,c] Same letter in the same row indicates absence of significant differences ($p < 0.05$).

wines (Carrascosa et al., 2012; De Quiros, Lage-Yusty, & Lopez-Hernandez, 2009), although others, such as flavonols and stilbene derivatives, found in previous studies, were only detected as trace levels in our study. Concentrations of all these phenolic compounds were significantly higher in the wines fermented spontaneously than in the wines elaborated with the commercial yeast strain (EC1118) or the recombinant strain (EKD-13), with the only remarkable exception of tyrosol (4-hydroxyphenylethanol), which exhibits an unusual higher value (143 mg/l, as average) in the wines elaborated with the recombinant EKD-13 strain. Data of individual flavan-3-ols, both flavan-3-ol monomers and procyanidins, also agreed with that of total catechins content (Table 3), all being significantly higher for the wines fermented spontaneously than for the wines inoculated with the selected yeast strains (EC1118 or its recombinant EKD-13). Flavan-3-ols, especially procyanidins, are largely responsible for the astringency and bitterness of the wine (Ribichaud & Noble, 1990).

4. Discussion

The recombinant EKD-13 yeast strain used in this work, has been previously used to obtain white wines from the Sauvignon-Blanc variety enriched in non-specific mannoproteins that help to protect against protein haze (Gonzalez-Ramos et al., 2008). However, its influence on the chemical composition of these wines, especially on the volatile and phenolic fractions, is still unknown. In this work, three different types of wine were prepared using must from the white Albariño variety, considered to be one of the top quality white varieties in Spain (Vilanova et al., 2010). A wine was obtained by spontaneous fermentation of the non-inoculated must, which is standard practice for many companies that use this grape variety. One wine was prepared by inoculating with the recombinant EKD-13 strain, and another with the EC1118 strain, a commercial yeast that was used as parental strain to develop the recombinant yeast EKD-13. In this yeast, in which both copies of the KRN4 gene are deleted, alterations in growth and in the fermentation performance have been described (Gonzalez-Ramos et al., 2008). Here, however, its fermentation performance was correct and no different to that recorded for the other wines obtained, in spite of the lower viability of EKD-13 than the other strains used from the middle phase of fermentation (Table 1). These results confirm those obtained by Gonzalez-Ramos et al. (2008), for fermentation of the Sauvignon Blanc must, who found that impairment of the fermentation performance in this strain was only minor and was detected only in the fermentation of musts with high-sugar content.

The study of the mannoproteins released into the wine confirmed the practical value of the recombinant strain EKD-13 to obtain mannoprotein-enriched wines. Gonzalez-Ramos et al. (2008) found that fermentation of the Sauvignon Blanc grape variety with this strain gave rise to a mannoprotein-enriched wine, determined by peroxidase-conjugated concanavalin A detection. Although this methodology cannot quantify the mannoproteins present in the wine, the authors found that these had a highly heterogeneous molecular weight, resulting from the nature of the genetic modification of the EKD-13 strain, designed to provoke release of mannoproteins from the cell wall. The analytical methodology used in this work quantified the polymeric mannose released into the wine, and found a value (593.5 mg/l), almost four times higher than 150 mg/l, which is the mean of the mannoprotein concentration in wines (Doco, Brillouet, & Moutounet, 1996).

Analysis of the main volatile compounds present showed that the most significant change observed in the wine made with the EKD-13 strain was associated with the increased concentration of 2-phenyl ethanol. This increase is due to the presence of the selection marker ARO4-OFP used during construction of the recombinant yeast. The ARO4 gene encodes the isoenzyme 3-deoxy-arabino-heptulosonate-7-phosphate synthase (DAHP), which is involved in the biosynthesis of aromatic amino acids, and its activity is sensitive to tyrosine (Braus, 1991). The mutated gene ARO4-OFP encodes for DAHP synthase isoenzymes and its activity is not inhibited by the presence of tyrosine (Fukuda, Asano, Ouchi, & Takasawa, 1992). Therefore, when this activity is suppressed, the yeasts overproduce phenylalanine, which is transformed by the Ehrlich pathway to 2-phenylethanol (Moreira, Guedes de Pinho, Santos, & Vasconcelos, 2011). Although we do not know the practical implications of the accumulation of 2-phenylethanol in this specific wine, it is interesting that in the group of higher alcohols this compound is the only one which, if present at higher concentrations than mean values reported in the literature (10–15 mg/l) (Armada, Fernández, & Falqué, 2010; Zamuz & Vilanova, 2006) could enhance the aroma of these types of wines, conferring them floral aromatic notes, which could be distinctive in white Albariño wines (Vilanova et al., 2010). On the other hand, in spite of their higher concentration of mannoproteins, an increase of varietal aroma compounds (terpenes and norisoprenoids) was not detected in wines made with the EKD-13 strain. In previous works it has been found that white wines made with the Albariño grape and a selected autochthonous yeast strain are richer in mannoproteins and varietal aromas than other similar wines (Carrascosa et al., 2012; Juega et al., 2012), and these colloid-rich

Table 4
Concentration (mg/l) of the main individual phenolic compounds in the different wines (non-inoculated fermentation, wines elaborated using EC1118 strain and wines elaborated using EKD-13 recombinant strain). Data are the mean ± SD ($n = 3$).

	Non-inoculated fermentation	EC1118	EKD-13
Gallic acid	3.36^{a} ± 0.11	3.28^{a} ± 0.08	3.29^{a} ± 0.07
Caffeic acid	2.71^{a} ± 0.18	2.68^{a} ± 0.08	2.44^{b} ± 0.05
Caftaric acid	12.7^{a} ± 0.3	10.6^{c} ± 0.7	11.3^{b} ± 0.1
p-Coumaric acid	11.90^{a} ± 0.09	1.11^{b} ± 0.15	1.10^{b} ± 0.01
Coutaric acid	5.15^{a} ± 0.34	4.39^{b} ± 0.18	4.53^{b} ± 0.05
Ferulic acid	3.65^{a} ± 0.03	2.90^{b} ± 0.04	2.96^{b} ± 0.03
Tyrosol	18.0^{b} ± 3.4	15.2^{b} ± 1.6	143^{a} ± 3.0
Tryptophol	4.95^{a} ± 0.10	4.96^{b} ± 0.23	4.52^{b} ± 0.08
(+)-Catechin	15.5^{a} ± 0.5	14.3^{b} ± 0.6	14.5^{b} ± 0.4
(−)-Epicatechin	8.89^{a} ± 0.76	7.32^{b} ± 0.59	7.12^{b} ± 0.29
Procyanidin B3	0.382^{a} ± 0.064	0.381^{a} ± 0.057	0.371^{a} ± 0.021
Procyanidin B1	1.20^{a} ± 0.15	0.895^{b} ± 0.071	0.904^{b} ± 0.034
Procyanidin B2	0.737^{a} ± 0.093	0.457^{b} ± 0.073	0.507^{b} ± 0.050
Procyanidin B7	0.368^{a} ± 0.061	0.317^{b} ± 0.043	0.327^{b} ± 0.032
Procyanidin C1	1.18^{a} ± 0.450	0.687^{b} ± 0.078	0.891^{ab} ± 0.065

^{a,b,c} Same letter in the same row indicates absence of significant differences ($p < 0.05$).

mannoproteins help to retain some varietal compounds such as geraniol and linalool (Juega et al., 2012). From the results obtained in this work it can be deduced that specific mannoproteins related to the yeast strain could be responsible for this behaviour, which does not depend on the final concentration of mannoproteins present in the wine.

The most significant influence of strain EKD-13 on the phenolic composition was associated with the high final concentration of tyrosol in the wines. Previous studies on Albariño wines reported values of tyrosol below 10 mg/l (Andrade et al., 2001; Carrascosa et al., 2012). At the concentrations normally found in wine, tyrosol seems to play little part in the wine aroma (Darias-Martín, Rodríguez, & Díaz, 2000). As said before for 2-phenylethanol, both tyrosol and tryptophol can be formed anabolically from sugars as well as catabolically from amino acids via the Ehrlich pathway, although it seems that formation of 2-phenylethanol and tyrosol mainly follows the Ehrlich pathway, whereas tryptophol is mainly produced anabolically from sugars (Garde-Cerdán & Ancín-Azpilicueta, 2008). Tyrosine (3-(4-hydroxyphenyl)-alanine) is the amino acid precursor for tyrosol and tryptophan (2-amino-3-(3-indolyl)-propionic acid) for tryptophol. For the recombinant EKD-13 strain, and as the mutant gene ARO4-OFP led to an amino acid overproduction, the Ehrlich pathway is activated and amino acids are transaminated to produce α-ketoacids that are further decarboxylated and reduced to alcohols. But this is only effective for 2-phenylethanol and tyrosol, but not for tryptophol, which would explain the results observed. The higher content of tyrosol in the wines elaborated with the recombinant EKD-13 strain is consequent with the higher values found for total polyphenols and TPI in these wines, as well as their higher browning potential (Table 3).

5. Conclusions

The recombinant EKD-13 strain managed to completely ferment musts of the Albariño grape variety in standard production conditions for these wines. The wines obtained presented some distinctive chemical characteristics, mainly derived from the modified gene expression in fermentation conditions. The most significant of these were the elevated mannoprotein concentration, and the final amount of 2-phenyl ethanol and tyrosol. Although some of these characteristics may be potentially useful from a technological point of view and distinctive from a sensorial perspective, further sensory evaluation is required to assess the real impact of these differences on the global perception of the wines obtained.

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CHAPTER 5

Effect of malolactic fermentation by *Pediococcus damnosus* on the composition and sensory profile of Albariño and Caiño white wines.

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CAPÍTULO 5

EFECTO DE LA FERMENTACIÓN MALOLÁCTICA REALIZADA POR PEDIOCOCCUS DAMNOSUS SOBRE LA COMPOSICIÓN Y EL PERFIL SENSORIAL DE VINOS BLANCOS ALBARIÑO Y CAIÑO DE LA D.O. RIAS BAIXAS

OBJETIVO

El objetivo principal del presente capítulo fue el de obtener cepas bacterias lácticas capaces de realizar la fermentación maloláctica e incrementar la tipicidad y mejorar la calidad en vinos de *Vitis vinifera* cv. Albariño y Caiño blanco de la D.O. Rías Baixas. En el momento de llevar a cabo este trabajo, no se había hecho estudio similar alguno sobre tal tipo de vinos.

PLAN DE TRABAJO

Para la realización del objetivo propuesto se llevaron a cabo las siguientes tareas:

1. Aislamiento de cepas de bacterias lácticas durante la fermentación maloláctica espontánea in vitro (1 L) de vinos de *Vitis vinifera* cv. Albariño y Caiño blanco y determinación, mediante métodos moleculares, de la especie y cepa a la que pertenecen, así como determinado su capacidad de producir aminas biógenas y exopolisacáridos para seleccionar y eliminar las no adecuadas.

2. Estudio de los vinos producidos mediante análisis instrumental, determinando su composición (grado alcohólico, extracto total seco, pH y dióxido de azufre total y libre, color, polifenoles totales y flavonoles).

3. Análisis sensorial de los vinos obtenidos.

RESUMEN

En este último capítulo de la tesis, se llevó a cabo la identificación y aislamiento de posibles bacterias lácticas autóctonas para conducir la fermentación maloláctica de vinos blancos Albariño y Caiño de la D.O Rias Baixas. A pesar de que este tipo de fermentación, es un proceso poco habitual en vinos blancos jóvenes de este estilo, su efecto en las características químicas, aromáticas y sensoriales de los vinos puede dar lugar a vinos con diferentes e interesantes atributos de tipicidad. Para este trabajo, vinos blancos de la variedad *Vitis vinífera* var. Caiño y Albariño no sulfitados tras la fermentación alcohólica, se inocularon con una cepa comercial de *Oenococcus oeni (O.oeni)* para arrancar la fermentación malolactica. El estudio de la secuencia del gen 16s rRNA en los vinos Caiño antes y después de la fermentación malolactica indicó la presencia de cepas de *Pediococcus damnosus* (*P. damnosus*) que parecían imponerse frente al resto de microbiota indígena y a la cepa comercial inoculada. Los resultados de RAPD PCR confirmaron la existencia de dos patrones de bandas de dos cepas diferentes, que eran las principales responsables de la fermentación maloláctica de estos vinos blancos Caiño y que pertenecían a la especie *P. damnosus* (cepas C5 y C8). En los vinos Albariño, ni la cepa de bacteria comercial ni la microbiota indígena fueron capaces de arrancar la fermentación malolactica después de 30 días. Con el fin de llevar a cabo esta fermentación, se inocularon las dos cepas aisladas de *P. damnosus* (C5 y C8). Los resultados del gen 16s rRNA y RAPD PCR indicaron la imposición de una de las cepas de *P. damnosus* inoculadas (C5) que resultó ser la principal responsable de la fermentación maloláctica de los vinos Albariño. Se comprobó además que las cepas aisladas de *P. damnosus* (C5 y C8) no poseían capacidad de producir aminas biógenas (ausencia de genes hdc, tdc y odc) y exopolisacáridos (ausencia del gen dps). Finalizada la fermentación maloláctica, se realizó un análisis sensorial comparativo para establecer las diferencias sensoriales entre los vinos. La realización de la fermentación maloláctica supuso un cambio en los resultados del

análisis sensorial. En los vinos de Albariño la fase visual presentó mayor intensidad de amarillos, en concomitancia con el descenso de acidez, y en la olfativa mayor intensidad en los descriptores de olor hierbas aromáticas y miel, apareciendo un nuevo descriptor (vainilla) y desapareciendo dos (pera y manzana). En la fase olfativa de los vinos de Caiño, los descriptores de frescor frutas tropicales, cítricos, banana, melocotón y pera desaparecieron, pero se identificó un nuevo descriptor (caramelo), aumentando significativamente el descriptor miel –que confiere a los vinos una mayor sensación de madurez- y disminuyendo hierbas aromáticas, así como la acidez y el amargor, este último sin significación estadística. Las cepas utilizadas sirvieron como una herramienta biotecnológica para modificar la calidad sensorial de los vinos estudiados.

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ORIGINAL ARTICLE

Effect of malolactic fermentation by *Pediococcus damnosus* on the composition and sensory profile of Albariño and Caiño white wines

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autochthonous *Pediococcus*, malolactic fermentation, sensory analysis, white wines.

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Abstract

Aim: This study was aimed to investigate the influence of malolactic fermentation (MLF) on sensory profile and organoleptic characteristics of Albariño and Caiño white wines.
Methods and Results: Autochthonous bacteria were isolated from wines after alcoholic fermentation (AF) and further identified as *Pediococcus damnosus* by 16S rRNA gene sequence analysis. When a commercial *Oenococcus oeni* starter was inoculated into Albariño and Caiño white wines to perform MLF, which was checked by HPLC quantification of malic and lactic acids, it was shown that autochthonous *Ped. damnosus* strains were able to predominate over the commercial *O. oeni* starter and perform MLF in Caiño wine. By contrast, neither commercial strain nor indigenous *Pediococcus* carried out MLF in Albariño wine. However, MLF was achieved when autochthonous strains that predominated in Caiño were inoculated into Albariño. Sensory analysis showed that after the MLF Albariño increased its body and softness, while Caiño result a more mature wine.
Conclusions: MLF can positively affect Albariño and Caiño wines giving them new attributes. Pediococci isolated and characterized in this work can successfully perform MLF without negative effects on the wine, because no production of biogenic amines or exopolysaccharides by the selected pediococcus strains was detected.
Significance and Impact of the Study: The effect of MLF in the sensory profile of Albariño and Caiño wines has never been studied before. Results obtained in this work showed that *Ped. damnosus* strains can be considered as a new topic of investigation on malolactic starter.

Introduction

The grape cultivar 'Albariño' is one of the oldest in the vine-growing area of Galicia in northwest Spain (Huetz De Lemps 1967). Until recently, this typical white grape variety was grown only for personal consumption, but nowadays, its area of production has increased enormously, being now one of the most prestigious grapes in Spain. Albariño wines are young balanced wines, fresh (due to a high acid content), and with an intense fruity and floral aroma (Dieguez *et al.* 2003; Oliveira *et al.* 2008; Armada *et al.* 2010).

The 'Caíño' cultivar group is considered to be the oldest grape variety in Galicia. Different types of 'Caíño' were first reported between 1909 and 1911 by García de los Salmones (1914). 'Caíño Blanco' is cultivated in the Rosal area, and it is used, like the Albariño, in the production of wines of recognized quality [Origin

Denomination (OD) Rías Consejo Regulador De La Denominación De Origen Rías Baixas 2004; Santiago *et al.* 2007].

The aroma is a very important component of the organoleptic quality of wine. The profile is influenced by different factors, environmental and management practices (Jackson and Lombard 1993), grape varieties (Schreier *et al.* 1976; Gunata *et al.* 1985), winemaking techniques (Dubourdieu 1986) and yeasts (Soumalainen and Lehtonen 1979; Carrascosa *et al.* 2012) among others.

Winemaking normally involves two fermentation processes, alcoholic fermentation (AF) by yeast, principally *Saccharomyces cerevisiae*, and a second fermentation, called malolactic fermentation (MLF), which can be performed by different lactic acid bacteria (LAB), generally associated with three genera: *Oenococcus*, *Lactobacillus* and *Pediococcus* (Maicas *et al.* 1999).

MLF, which is conducted in most red and in some white wines, is not usually accomplished in Albariño wines, characterized by a typical fresh taste. The activity of the LAB that perform MLF decreases wine acidity through the conversion of the dicarboxylic malic acid to the monocarboxylic lactic acid. MLF produces a number of compounds that modify flavour, being diacetyl one of the most recognized. Other compounds produced by LAB metabolism are 1-hexanol, ethyl acetate, ethyl lactate, diethyl succinate, γ-butyrolactone, glycoaldehye, glyoxal, 2,3-butanediol, and caprylic acid. The synthesis of these aromatic compounds promotes the reduction of vegetable and herbaceous aromas and the appearance of other fruity and floral aromas in wines (Sauvageot and Vivier 1997).

To obtain a more complex aroma, and also for the microbiological stabilization of wine, MLF is also conducted in some white wines. Moreover, LAB seems to have glycosidase activity, and they are able to release terpenes, norisoprenoids, phenols and vanillin from grape glycosidic precursors (Hernandez-Orte *et al.* 2009).

In most cases, *Oenococcus oeni* is the only species identified when the MLF is completed (Lonvaud-Funel *et al.* 1991), as it is the best adapted LAB to the difficult growing conditions (low pH, high ethanol content, and the presence of SO_2) that characterize the wine. *Oenococcus oeni* is present in wine during AF together with other LAB belonging to *Pedioccocus*, *Leuconostoc* and *Lactobacillus* genera but, generally, towards the end of the AF, *Lactobacillus*, *Pediococcus* and *Leuconostoc* species progressively disappear (Lonvaud-Funel 1999).

Lactobacillus and *Pediococcus* are generally considered spoilage bacteria in wine, because some strains can synthesize exopolysaccharides and, consequently, provide a viscous and thick texture to the wine (Walling *et al.* 2005). Moreover, they can also produce high concentrations of acetic acid (Lafon-Lafourcade *et al.* 1983) and biogenic amines (BA) (Landete *et al.* 2005).

However, as previously suggested (Fugelsang and Edwards 2007), and as these negative properties are strain-related, the growth of lactobacilli and pediococci in wine may not necessarily affect adversely its quality but, on the contrary, may add desirable flavours and aromas under certain circumstances. In fact, because *Lactobacillus* and *Pediococcus* species possess a high number of enzyme encoding genes important for the production of wine aroma compounds, their use as starter cultures to carry on MLF has recently been investigated (Du Toit 2011; Strickland 2012).

This work reports the first study on the influence of MLF on sensory profile and organoleptic characteristics of Albariño and Caiño wines. Moreover, the native LAB strains of these white wines were investigated. Our study showed that MLF was accomplished by two indigenous *Pediococcus damnosus* strains that predominate over the inoculated *O. oeni* strain and that they did not present negative properties eventually related with the *Pediococcus* genus. Chemical and physical parameters and sensory analysis were performed before and after MLF to examine and evaluate the effect of this process on the sensory attributes of the wines. Results obtained show that the strains of *Ped. damnosus* isolated here are suitable starters for MLF in white wines and that MLF can positively affect Albariño and Caiño wines.

Materials and methods

Wine samples

A total of 12 L of Caiño and Albariño wines (vintage 2009) fermented in a winery located in the zone of the O Rosal in the province of Pontevedra in Galicia (Spain), pertaining to the Rías Baixas O.D., was used in this study. Samples were taken before and after MLF, and no SO_2 was added during the process.

Wine composition analysis

Wines were analysed both before and after the MLF for the following chemical parameters: alcohol content, total dried extract, pH and total and free volatile SO_2, according to official methods (G.U.C.E. n. 272 3/10/1990). The colour of wine was also tested using CIE-CIELAB parameters. Fixed acids were analysed by HPLC according to Cane (1990). Phenol composition (total polyphenols and flavonoids reaction to p-DACA) was determined according to Di Stefano *et al.* (1989). Physical and chemical composition of Albariño and Caiño wines, before (initial wine) and after MLF, is shown in Table 1.

Table 1 Physical and chemical parameters of wines before and after MLF

	Albariño before MLF	Albariño after MLF	Caiño before MLF	Caiño after MLF
Current assays				
Alcohol (%)	12·98 ± 0·04	13 ± 0·03	12·46 ± 0·02	12·46 ± 0·01
pH	3·51 ± 0·00	3·68 ± 0·00	3·71 ± 0·00	3·79 ± 0·01
Total acidity (g/l)	7·4 ± 0·00	5·2 ± 0·00	6·65 ± 0·07	5·00 ± 0·00
Volatile acidity (g/l)	0·49 ± 0·03	0·525 ± 0·02	0·36 ± 0·01	0·41 ± 0·01
Free SO_2 (mg/l)	6·40 ± 0·00	4·8 ± 0·00	6·24 ± 0·22	3·40 ± 0·85
Total SO_2 (mg/l)	42·0 ± 4·24	41·0 ± 1·41	40·0 ± 1·41	37·9 ± 2·83
Colour				
E 420 nm	$0{\cdot}17 \pm 4{\cdot}1 \times 10^{-3}$	$0{\cdot}19 \pm 2{\cdot}1 \times 10^{-4}$	$0{\cdot}11 \pm 7{\cdot}07 \times 10^{-5}$	$0{\cdot}16 \pm 3{\cdot}54 \times 10^{-4}$
CIE				
Brightness Y%	$0{\cdot}94 \pm 4{\cdot}36 \times 10^{-3}$	$0{\cdot}93 \pm 8{\cdot}37 \times 10^{-5}$	$0{\cdot}96 \pm 1{\cdot}5 \times 10^{-3}$	$0{\cdot}92 \pm 1{\cdot}46 \times 10^{-3}$
Saturation S%	11·71 ± 0·16	13 ± 0·01	7·74 ± 0·06	$9{\cdot}78 \pm 3{\cdot}67 \times 10^{-3}$
CIELAB				
*L**	97·66 ± 0·17	$97{\cdot}31 \pm 3{\cdot}4 \times 10^{-3}$	98·56 ± 0·06	$96{\cdot}94 \pm 2{\cdot}62 \times 10^{-4}$
*a**	$-2{\cdot}75 \pm 3{\cdot}04 \times 10^{-3}$	$-2{\cdot}44 \pm 2{\cdot}4 \times 10^{-3}$	−2·15 ± 0·03	−1·97 ± 0·06
*b**	12·71 ± 0·15	13·95 ± 0·01	8·57 ± 0·07	10·51 ± 0·01
*h**	$-1{\cdot}36 \pm 2{\cdot}23 \times 10^{-3}$	$-1{\cdot}40 \pm 3{\cdot}1 \times 10^{-4}$	$-1{\cdot}33 \pm 1{\cdot}9 \times 10^{-3}$	−1·38 ± 0·01
*C**	13·00 ± 0·14	14·17 ± 0·01	8·83 ± 0·08	10·69 ± 0·02
Phenolic composition (mg/l)				
Total phenolic content	107·00 ± 5·65	106·00 ± 1·41	97·50 ± 0·70	96·50 ± 3·54
Flavonoids reaction to p-DACA	65·00 ± 1·41	63·00 ± 1·41	54·50 ± 0·70	52·00 ± 1·41

MLF, malolactic fermentation.

Malolactic fermentation assays

To study the effect of MLF on these wines, they were inoculated with a commercial *O. oeni* starter (Lallemand, Italy) normally used in the winery. This dried commercial strain was rehydrated for 20 min in distilled water at 30°C and employed following manufacturer's instructions and then inoculated in 5 l of either Albariño or Caiño wines.

As no MLF was observed in Albariño wine, a second inoculation (10^6 cell ml^{-1}) was performed with a mix of two indigenous strains of *Ped. damnosus* isolated from Caiño wine that had been previously grown in a wine-like medium (MRS diluted 1 : 10 with water and added with 3 g l^{-1} malic acid and 12% ethanol). Wines were incubated at 25°C until consumption of malic acid, which was quantified by HPLC (Waters Binary HPLC 1525 pump, Turin, Italy) using a RP 18 5 μm column (LiChrospher® 100; Merck KGaA, Darmstadt, Germany) as previously described (Cane 1990). External standards were purchased from VWR (Milan, Italy).

To confirm the results obtained from these fermentations, new tests were made by utilizing Caiño wine previously sterilized by filtration with 0·2-μm filter. Three tests were performed, in duplicate, with the selected *Pediococcus* and the commercial *Oenococcus* strain: (i) The inoculum for MLF was made with *Ped. damnosus* mix, (ii) The inoculum for MLF was made with *O. oeni*, and (iii) The inoculum for MLF was made with *Ped. damnosus* mix *plus O. oeni*. The level of inoculum was 10^6 cell ml^{-1} for both species. Malic acid consumption was monitored by HPLC as described previously.

Isolation and characterization of the LAB

Microbial strains and growth conditions

Samples of Caiño and Albariño wines, taken before and after MLF, were serially diluted in 0·9% (w/v) NaCl and 1 ml aliquots were plated on MRS agar, pH 4·8, (VWR) with 0·1 mg ml^{-1} of cycloheximide (Sigma-Aldrich, Milan, Italy) to suppress yeast growth. After anaerobical growth at 30°C, 50 colonies were randomly selected and grown individually in liquid MRS, pH 4·8, supplemented with 10% (v/v) tomato juice (Difco, BD, Milan, Italy), and further incubated at 30°C. LAB cultures were maintained at −80°C in the isolation medium supplemented with glycerol 50% (v/v).

DNA extraction

DNA was extracted with 'Archive pure DNA, Yeast and Gram+ kit' (5-PRIME, Hamburg, Germany). Cultures were centrifuged (15 000 ***g***, 1 min), and the pellet was treated according to the kit manufacturer's instructions. DNA samples were measured in a spectrophotometer (BECKMAN COULTER DU® 730, Life Science UV/VIS, spectrophotometer, Beckman Coulter, Milan, Italy) at 260–280 nm.

Species identification
DNA was amplified with universal primers 63f-1387r (Marchesi *et al.* 1998) and sequenced (BMR-genomics, Padova, Italy). Sequence similarity searches were performed using the *Blast* algorithm (Altschul *et al.* 1990) at NCBI (www.ncbi.nlm.nih.gov) on the GeneBank databases and the Ribosomal Database Project (Cole *et al.* 2009).

Strain typing
RAPD-PCR was performed with primers Coc and On2 (Cocconcelli *et al.* 1995; Reguant and Bordons 2003) at 95°C for 5 min, followed by 30 cycles at 94°C for 1 min, 40°C for 1 min and 72°C for 2 min, with a final extension step of 10 min. Amplified products were visualized by ethidium bromide staining after gel electrophoresis. The marker used was 1Kb DNA Ladder (Sigma, Milan, Italy).

The band pattern obtained were compared using Bionumerics (Applied Maths, Ghent, Belgium) Dice's similarity coefficients were calculated as $2n/a+b$, where n = the number of matching bands and $a + b$ = the total number of bands (matching and no matching), and strains were grouped by using the unweighted pair group method with arithmetic averages (UPGMA); the profile of the commercial strain inoculated was used for comparison.

Determination of the biogenic amines-producing capability
The presence of decarboxylase genes *hdc*, *tdc* and *odc* was assessed by PCR according to Costantini *et al.* (2006), and the capability to produce histamine, putrescine and tyramine was determined by TLC according to Garcia-Moruno *et al.* (2005).

Evaluation of the capability to produce exopolysaccharides
PCR assay was performed according to Walling *et al.* (2005) using primers PF1/PF8 that amplify the *dps* gene, which product is responsible for the glucan biosynthesis.

Sensory evaluation

The wines were evaluated before and after the MLF in two replicates by a CRA-ENO trained panel composed by 14 experts (seven male and seven female) aged 25–60 years in an ISO (8589-2007) tasting room. The procedure followed derived from the ISO standards (11035-1994). Firstly terms, describing colour, odour and flavour (taste and mouth-feel sensations) of the wines were collected. Afterwards, the intensity of the chosen descriptors was measured using an unstructured intensity scale ranging from 0 to 80 mm presented on a wheel. Wine samples (30–40 ml) at 10–12°C were poured in ISO (3591-1977) approved glasses immediately before analysis and covered by Petri dishes. Samples were presented in a random order with a three-digit code.

Data were analysed by two-way analysis of variance (ANOVA) using XL Stat software (Addinsoft, Milan, Italy). Mean comparisons were performed by Tukey's test at $P < 0{\cdot}05$. The data processed were represented by the common descriptors of the wines before and after MLF and the repetition of the sessions. For both wines, Albariño and Caiño, the ANOVA analysis made on the replications of the sensory session showed that there were not significant differences, and thus, the average of the data of the two replicates were considered.

Results

Development of MLF

Wines after AF were not sterilized to simulate the real conditions of the winery and were inoculated with an *O. oeni* commercial starter (about 10^6 cell ml^{-1}) as described in material and methods; the MLF was considered concluded when the malic acid was totally consumed. The commercial starter was chosen because it is a strain currently utilized in wine industry and, according to the manufacturer, suitable for white wine because it points out fruity aromas.

In Caiño wine, the bacterial population after the AF, before the inoculation of the starter, was 4×10^2 cell ml^{-1} and was constituted by *Ped. damnosus*, as shown after 16S rRNA gene sequence analysis. In this wine, MLF was completed in 30 days (Fig. 1). To verify whether the inoculated *O. oeni* commercial starter was responsible for the MLF, bacteria present in the wine at the end of MLF (2×10^5 cell ml^{-1}) were isolated and characterized. Results showed that *O. oeni* commercial strain did not conduct the MLF, as 16S rRNA gene sequence analysis showed that only pediococci, identified as *Ped. damnosus* with an identity of 99%, were isolated. RAPD PCR typing of the strains showed two different band patterns. Comparison of the band profiles showed that the two biotypes

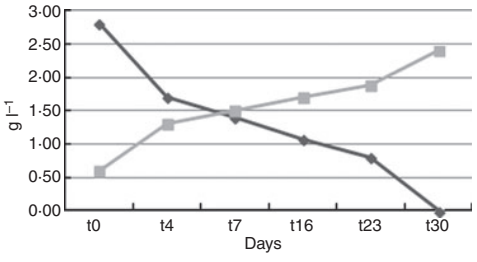

Figure 1 Consumption of malic acid and formation of lactic acid during malolactic fermentation in Caiño wine. Inoculation of commercial *Oenococcus oeni* starter at $t = 0$. (—◆—) malic ac; (—■—) lactic ac.

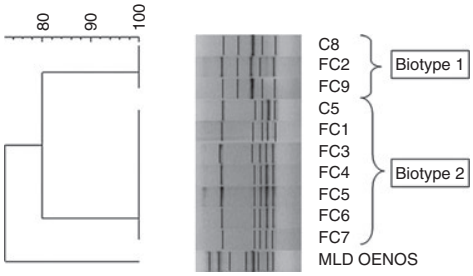

Figure 2 Dendrogram of patterns of *Pediococcus damnosus* isolated from Caiño wine after malolactic fermentation (MLF) showing the presence of two different biotypes. MLD OENOS, commercial strain. C5 and C8, autochthonous *Ped. damnosus* strains chosen as representative of each biotype. FC, bacteria isolated at the end of the MLF.

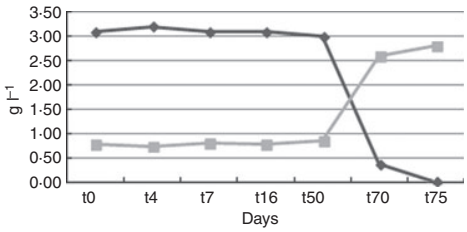

Figure 3 Consumption of malic acid and formation of lactic acid during malolactic fermentation in Albariño wine. Inoculation of commercial *Oenococcus oeni* starter at $t = 0$. Inoculation of autochthonous *Pediococcus damnosus* mix (C5 and C8) isolated from Caiño wine at $t = 45$. (—◆—) malic ac; (—■—) lactic ac.

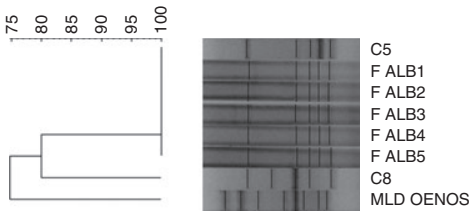

Figure 4 Dendrogram of patterns of *Pediococcus damnosus* isolated from Albariño wine after malolactic fermentation (MLF) (indicated as FALB). MLD, commercial *Oenococcus oeni* strain. C5 and C8, indigenous *Ped. damnosus* strains isolated from Caiño wine that was inoculated in Albariño wine. FALB, bacteria isolated at the end of the MLF.

present at the end of MLF were also present in the initial wine (Fig. 2). Therefore, these indigenous strains were able to predominate over the commercial *O. oeni* bacterial starter. Two strains, C5 and C8, were chosen as representative of the two identified biotypes.

In Albariño wine, bacterial indigenous population after AF was 50 cell ml^{-1}, and it was constituted by *Ped. damnosus*. MLF using commercial starter was not initiated after more than one month of incubation; therefore neither the *O. oeni* starter, nor the indigenous population were capable to predominate and start MLF. Wine was then inoculated (at the 45th day) with a mix of the two autochthonous *Pediococcus* strains isolated in Caiño wine (C5 and C8). Under these conditions, MLF was also finished in Albariño wine in less than 30 days after inoculation (Fig. 3). The band profiles of the colonies isolated at the end of MLF, obtained by RAPD PCR analysis, showed that they were grouped to only one of the two strains inoculated, the strain C5 (Fig. 4).

These results confirmed that the autochthonous strains isolated in Caiño wine were well adapted to the production conditions of both Albariño and Caiño wines, particularly C5 strain that seems to be better adapted than C8 to the conditions of Albariño wine.

Further tests were made on Caiño wine that had been sterilized and inoculated as described in Materials and methods. After 35 days, MLF was only completed in those tests in which inoculation was performed with *Pediococcus* alone or with *Pediococcus plus Oenococcus*, respectively, while no MLF was observed in those only inoculated with *Oenococcus*. These results confirmed that *Pediococcus* is better adapted to these white wines. Moreover, these data showed that the failure of *O. oeni* to perform MLF in the wines is not due to a competition with the autochthonous *Pediococcus*, as in these test, the wine had been previously sterilized.

The main concern related with some *Pediococcus* strains is the production of glucan, an exopolysaccharide that causes an alteration of the wine called 'ropiness'; therefore, to evaluate the possible utility of these autochthonous strains as starters for MLF, the presence of *dps* gene, responsible for the glucan biosynthesis, was assayed by PCR. No amplification was achieved, indicative of the absence of these genes in the strains tested (data not shown).

Another concern is the production of BA, which are undesirable in all foods and beverages, as they can induce headaches, respiratory distress, hyper/hypotension and several allergic disorders (Ladero *et al.* 2010). BA are produced by some LAB by decarboxylation of precursor amino acids such as histidine, tyrosine, and ornithine, resulting in the formation of histamine, tyramine and putrescine, respectively, which are the most frequently BA found in wine (Lonvaud-Funel 2001; Ancín-Azpilicueta *et al.* 2008). PCR results demonstrated that the indigenous strains isolated in Caiño wine did not possess genes

codifying for decarboxylase enzymes (*hdc*, *tdc*, *odc* genes). This result was also confirmed by TLC analysis, as production of BA by the studied bacteria in the presence of the BA precursor amino acids was not detected (data not shown).

Modification of wines after MLF

As displayed in Table 1, the initial total acidity was elevated in both wines. This acidity is characteristic of wines that are consumed young, as the ones studied here. After MLF, there was a moderate increase in pH values, but an important decrease in total acidity, which transformed the typical organoleptic characteristics of these wines.

Regarding colours parameters, it can be observed (Table 1) that in the case of Albariño wine the E420, S% and *C** parameters had a light increase after MLF, meaning that the colour became more intense. The green component (*a**) of the colour decreased, while the yellow one (*b**) increased after MLF. This was observed also at a sensory level: the intensity of the yellow-golden highlights increased significantly (Fig. 5), while the transparency of the wine after MLF did not change (see Y% and *L** values).

For Caiño wine, the colour was less intense than in the initial Albariño wine (at the end of the AF) and, after the sensory analysis, the colour was described by tasters as 'straw yellow with yellow highlights'. After MLF there was, even in this case, an increase in the colour intensity (see E420, S% and *C** values), but this was less evident for the panel (Fig. 6). There was also a decrease on the transparency of the wine (see Y% and *L** values). On the other hand, phenolic composition of both wines was not affected by MLF.

The sensory profile of Albariño wine before MLF showed a straw yellow colour with yellow-golden highlights, all the odour descriptors – acacia flowers, citrus fruits, pineapple, pear, apple, aromatic herbs and honey – had a high intensity, the acidity was evident, the bitterness low, and it was considered a soft and high body product.

Figure 5 shows the evaluations of the common descriptors after comparison of the Albariño sensory profiles before and after MLF. The only significant difference was found for yellow-golden highlights, which increased after MLF as a consequence of the decrease in the acidity. Other differences, although not statistically significant, were observed for the odour descriptors: aromatic herbs and honey increased after MLF, while pineapple decreased. Two fruity odour descriptors, pear and apple, disappeared and the new descriptor vanilla were identified. A little improvement was reported for flavour descriptors, softness and body increased.

In the case of Caiño wine, before MLF the odour was characterized by high intensity of flowers (acacia flowers and orange blossom), tropical fruits, citrus fruits, banana, peach, pear, honey and aromatic herbs. As in the case of Albariño, in this product, the acidity was evident, the bitterness low, and it was considered a soft and high body product. It was shown that in Caiño, after MLF, 'fresh' notes such as tropical fruits, citrus fruits, banana, peach and pear disappeared, but a new odour descriptor

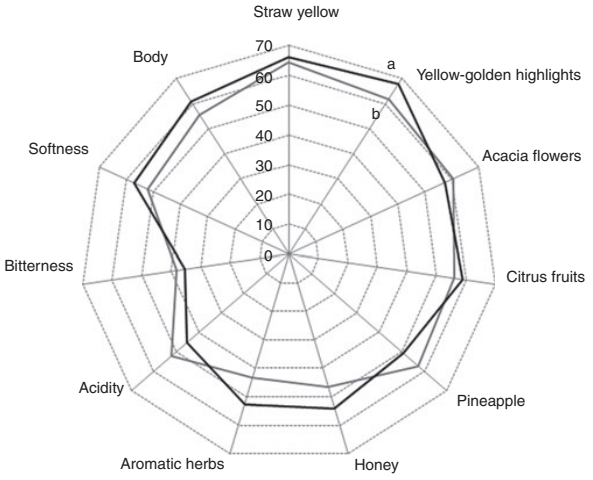

Figure 5 Comparison of the sensory profile of Albariño before and after malolactic fermentation using common descriptors. a and b indicate significant differences (ANOVA and Tukey's test, for $P = 95\%$).

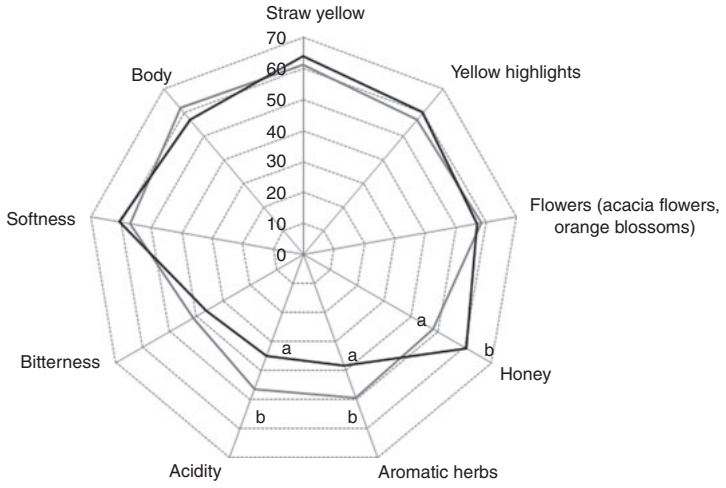

Figure 6 Comparison of the sensory profile of Caiño before and after malolactic fermentation using common descriptors. a and b indicate significant differences (ANOVA and Tukey's test, for $P = 95\%$).

caramel was identified. Statistical analysis performed on the common descriptors showed significant differences for acidity, aromatic herbs and honey (Fig. 6). Acidity decreased after MLF. Honey increased in wine after MLF, and this aspect gave to the wine more 'mature' notes, while aromatic herbs decreased. A not significant bitterness decreased was also observed after MLF.

Discussion

MLF is considered beneficial to the quality of the finished wine, and it is influenced by various factors. Principal among them is the presence and quantity of indigenous LAB that may include species from the genera *Lactobacillus*, *Oenococcus*, *Pediococcus* and *Leuconostoc* (Wibowo *et al.* 1985; Bae *et al.* 2006; Konig *et al.* 2009).

The selection of bacterial strains for the vinification process is principally based on its compatibility with the wine environment and the consumption of malic acid. In this regard, various works have been focused on the influence of different conditions, such as high ethanol concentration, low pH, or SO_2 concentration on MLF and bacterial activity (Gockowiak and Henschke 2003; Alegria *et al.* 2004; Guzzon *et al.* 2009).

In white wines, MLF is not always carried out, because white wines are characterized by fresh notes and acid taste and, thus MLF, which reduces acidity, can be not desired for its effects. Consequently, Albariño and Caiño wines are usually bottled after AF without performing MLF. However, MLF could add beneficial flavour and, hence, starting from the same grapes, it could be possible to produce wines with different characteristics. For instances, an increase in the content of monoterpene, acetates and volatile phenols after MLF have been described in Albariño commercial wines from different origins (Falqué *et al.* 2008).

In the present report, the influence of MLF on sensory profile and organoleptic characteristics of Albariño and Caiño wines has been addressed for the first time. Microbiological characterization has shown that they contained bacteria belonging to *Ped. damnosus* species. Inoculation of these wines, which were not sterilized to simulate real winemaking conditions, with a commercial *O. oeni* starter to perform MLF showed that in Caiño wine MLF was accomplished by two indigenous *Ped. damnosus* strains that predominated over *O. oeni* starter, probably because the wine had a relatively high pH. By contrast, in Albariño neither the commercial *O. oeni* starter inoculated at the end of AF, nor the autochthonous population accomplished MLF, which was only completed after inoculation of *Ped. damnosus* strains isolated from Caiño wine. It is possible that the lack of spontaneous MLF in Albariño wine could be due to a low level of native bacteria at the end of AF. These data indicate that in these wines, which are characterized by high pH, the selected *Ped. damnosus* strains are particularly suitable starter for MLF.

This hypothesis was confirmed by subsequent tests conducted on sterilized Caiño wine, which showed that while MLF was not performed by *Oenococcus*, the selected autochthonous *Ped. damnosus* successfully do it, both alone and in the presence of *O. oeni*. Thus, these analyses conducted in sterilized wine that avoid the presence of active *Pediococci* demonstrated that the autochthonous *Pediococci* do not inhibit *O. oeni* growth, but that the specific wine characteristics are more probably responsible

for the incapacity of *O. oeni* to complete MLF, even though this strain is a commercial starter widely used in the wineries. These results confirm that the selected *Pediococcus* strains are better adapted to this wine.

Pediococcus damnosus, *Leuconostoc mesenteroides*, and *O. oeni* have been identified as the key LAB accountable for MLF (Lonvaud-Funel 1999), but wine pH strongly influences which LAB species will be present. Higher pH wines (above pH 3·5) often harbor species of *Lactobacillus* and *Pediococcus*, both during and after fermentation, while lower pH wines (<3·5) typically contain only *O. oeni* (Fleet 1998; Osborne and Edwards 2005). In fact, the frequent occurrence of *Pediococcus* spp. in Australian wines has been proposed to be indicative of a high concentration of SO_2 and a high pH (Costello *et al.* 1983), as *Ped. cerevisiae* (now *Ped. damnosus)* was dominant in the wines analysed (45 reds and 24 whites) and *O. oeni* population disappeared. Likewise, Davis *et al.* (1986) also showed that the pH had a profound, selective effect upon the species that grow in wines, as *O. oeni* was usually the only one isolated from wines with a pH below 3·5, in which *Pediococcus* and *Lactobacillus* spp. rarely grow, although they do it in wines with pH over 3·5 (Ribereau-Gayon *et al.* 1975; Davis *et al.* 1986). Thus, growth of these bacteria seems to be antagonistic to *O. oeni* survival (Wibowo *et al.* 1985; Davis *et al.* 1986). Chemical and biomolecular assessment of the possible negative characteristics usually attributed to *Ped. damnosus* species of the autochthonous strains isolated here from Caiño wine demonstrated that they did not produce BA or exopolysaccharides.

Once the lack of the above-mentioned negative characteristics of the selected *Pediococcus* strains was verified, the wines were evaluated with sensory analysis by a trained panel of tasters before and after MLF. This analysis indicates that the MLF induced in Albariño both an aromatic change and a significant difference in the colour, which became more yellow-golden, as well as a decrease in the acidity. Moreover, after MLF, there was an increase in the body and softness of this wine. On the other hand, Caiño wine that underwent MLF was described as more mature than the original one showing a lower acidity.

The presence of volatile compounds and sensory analyses of Albariño musts and wines have been already reported (Oliveira *et al.* 2008; Vilanova *et al.* 2008, 2010), but, until now, no previous studies on the effect of MLF on this wine or on the sensory profile of Caiño wine has never been made. The analysis of these profiles showed that MLF gave new characteristics to the wines without affecting flower descriptors. Therefore, and even though, to maintain their fresh notes, MLF is not usually allowed to occur in white wines, on the bases of the results present in this study, it can be useful to wine industry for the production of wines having more complex characteristics.

In the last two decades, and albeit the use of malolactic starter cultures has become widespread to carry on the MLF, the homofermentative species *Ped. damnosus* has not being used because it is considered a spoilage microorganism. However, our results indicate that the strains isolated in this report are useful starters for the MLF in wines that, as Albariño and Caiño, are characterized by a high pH value. Supporting this, it has been reported that MLF significantly contribute to the formation of volatile aroma compounds in white wines, as Chardonnay, and that pH and ethanol content were important factors influencing the volatile aroma composition (Knoll *et al.* 2011). Even more, the same study also suggest that the choice of the starter is very important to obtain a wine with the desired final characteristics, as it can influence the final chemical composition and the sensory profile of the wine. Likewise, several strains of *Pediococcus* spp were isolated from wines that were not considered spoiled, but on the contrary they were judged to be of good quality (Edwards and Jensen 1992).

As commented previously, there is an increased interest to investigate new species for MLF. In this respect, and although *Pediococcus* is usually considered a spoilage microorganism together with *Lactobacillus* species, some researchers have isolated *Pediococcus* from wines that were not considered spoiled. For example, *Pediococcus parvulus* altered the bouquet of a non-MLF Cabernet Sauvignon, but did not spoil it (Osborne and Edwards 2005), thus confirming that some pediococci may add desirable flavours and aromas to the wine. In agreement with that, our results indicate that in wines with high pH, as Albariño and Caiño, *Pediococcus* is well adapted to perform MLF. Even more, under a practical point of view, an important characteristic of *Pediococcus* is its higher growth rate, making its employment an interesting option in winemaking.

In conclusion, this study demonstrated that MLF can positively affect Albariño and Caiño wines, and that the bacterial indigenous population of these wines was composed by *Pediococcus*, which can successfully perform MLF without negative effects on the wine, as they did not produce BA or exopolysaccharides. Therefore, *Ped. damnosus* strains can be considered as a new topic of investigation on malolactic starter.

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Conflicts of Interest

No conflict of interest declared.

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GENERAL DISCUSSION

General discussion

Although a specific discussion has been presented in each chapter, a brief general discussion linking the results obtained in each of the previous chapters is presented in this section to provide a general overview of the study done along the PhD thesis. In this sense, the discussion is organised according the presentation of the articles in the present report, summarizing the main activities developed and the results obtained in each case.

1. Improvement of final quality in Albariño wine

Galicia (NW Spain) is a wine-producing region in which the production of quality wines (PDO wines) has a special economic importance. To achieve a PDO brand, wines must be made of only those grape varieties authorised by the Regulator Council; furthermore, the berries must be grown in vineyards located in a controlled geographical area. Rias Baixas area is a restricted wine-growing region in Galicia where *Vitis vinífera* cv Albariño is the main white variety for the production of typical fresh, intense floral and fruity Albariño wines. *Vitis vinífera* cv white Caiño is other traditional Galician grape variety that may be used in a minor proportion in Rias Baixas PDO. The special aromatic characteristics of both V*itis vinífera* cv Albariño and Caiño white varieties make wines from Rias Baixas PDO to be high quality wines.

Aroma is considered one of the most important properties in Albariño wines, and the main determinant of quality. Presently, several hundred volatile aroma compounds such as alcohols, esters, organic acids, aldehydes, ketones and terpenes have been identified in wine, and their combination contribute to form the character of wine and the possibility to differentiate one wine from another. Over 800 volatile compounds have been found in wines, with a wide concentration range varying from hundreds of mg/L to the µg/L or ng/L level (Li, 2006).

Another chemical compounds that in the last years have increased their importance in wineries are phenolic compounds. These compounds are partly responsible for the colour, astringency, bitterness, and aroma of wine (Kennedy, 2008) In white wines, the

concentration of these compounds is lower than in red wines although their effect in aroma and wine browning process is especially important.

Strategies focused in the improvement of the aromatic character and phenolic composition of these wines result in an increase in final wine quality and in a greater appreciation by the customers. For this reason, the main approach of the current thesis was to improve the final quality of Albariño wines through the use of biotechnological and microbiological tools.

Most of the experiments performed along the current thesis were carried out both in laboratory and real winery conditions.

2. Influence of locally selected yeast on the chemical and sensorial properties of Albariño white wines.

Spontaneous alcoholic fermentation of grape must that benefit from indigenous microorganisms is a widespread practice in many wineries from Albariño PDO. This is considered by many grape growers as an option to ensure the expression of typical characteristics of variety associated to its terroir. Nevertheless, sometimes this practice has negative effects: the lack of homogenity between vintages, the dependence of adverse weather conditions or unexpected defects in wines. For this reason, the use of commercially available yeast strains to inoculate grape musts has become a posible alternative option. However, apart from the technological benefits obtained, the use of commercial dried yeast reduce typical characteristics of wines and possibly their complexity regardless the winemaking location. For this reason, the first objective of the current thesis was the selection of an autochthonous yeast strain to carry out the fermentation of Albariño white wines, to both control the alcoholic fermentation and enhance typical characteristics of wine variety.

Due to the importance of *Saccharomyces cerevisiae* (*S. cerevisiae*) strains in alcoholic fermentation and it´s effect in the final characteristics of wine, in a first study, we inquired into the effect of using different selected indigenous strains, isolated and collected from Albariño musts along several years, in the alcoholic fermentation of Albariño grape juice. Three indigenous *S.cerevisiae* strains (1, 2 and 3) were inoculated in Albariño musts to

determine the their influence in the sensorial characteristics, and volatile and phenolic profile of final wines (W1, W2 and W3). The results showed that these locally selected yeasts inoculated as a single inoculum in the same Albariño musts, significantly affected the fermentation and influenced the volatile and phenolic profile of the resulting wines. Those Albariño wines inoculated with *S. cerevisiae* 1 were qualified from tasters as "very good" in contrast with the rest of wines considered as "correct". Furthermore, Albariño wines fermented with *S. cerevisiae* 1, presented the most interesting chemical properties due to a higher concentration of terpenes and norisoprenoids, the main responsible of the fruity and fresh character of Albariño wines. Moreover, the concentration of flavan-3-ols, closely related with astringency and bitterness, was significantly lower and they presented the lowest browning potential, which could help to preserve some of its sensorial properties over time. As a result of this study along with the Galician Biological Mission, the use of indigenous *S.cerevisiae* strain 1 was patented to carry out the fermentation of Albariño white wines from Rias Baixas PDO. This patent was entitled to the Spanish winery Terras Gauda, a private company that is currently exploiting it (**Annexe 1**). In this winery, aproximately 1000000 liters of wine per year are inoculated with the *S. cerevisiae* 1 strain isolated and identified in this first study. This strain is provided as a culture starter by a spin off company arising from CSIC, Biopolis. Apart from these results, which indicate the practical application of this study, most relevant scientific results are gathered in the article 1 of the current thesis. These results with those obtained in a viticulture study of the Albariño variety and those published in "Study to improve the Albariño wine quality", were awarded in 2009 by the Royal Galician Academy of Science (**Annexe 2**).

3. Influence of yeast mannoproteins in the aroma improvement of white wines.

One of the most distinctive characteristics of the selected autochthonous *S. cerevisiae* 1 strain is its capacity to improve the concentration of terpenes and norisprenoids in wines. These are varietal compounds synthesized by grape metabolism. Often, they are present in grapes as glycosidically bound flavorless precursors. Therefore, the effect of the yeast strain in the concentration of terpenes and norisprenoids in wines could be due to 2 main reasons: the synthesis of β-glucosidases enzymes to hydrolyze sugar bound in aroma precursors and the release of mannoproteins that interact with aromatic compounds.

In the second part of this study the main objective was to determine which of these holdings characterize *S. cerevisiae* 1 strain. The experiments, which were done with different indigenous pre-selected strains (strains 1, 2, and 3), showed that none of them were able to synthetize and release β-glicosidases into the wine (data not shown). For this reason, the ability of these strains to release manoproteins related with the aromatic composition, especially varietal compounds, was determined.

In the last few years, wineries were allowed by the OIV in the resolution C.19/2006 to use mannoproteins from yeast in white and red winemaking. These colloids are one of the main components presented on yeast cell walls and have been associated with some positive oenological properties among which highlight it's ability to interact with aromatic compounds improving the aroma profile of wines. In this research, mannoprotein and volatile compounds were measured in inoculated wines with *S.cerevisiae* strains 1, 2 and 3. Wines fermented with the strain *S.cerevisiae* 1 showed both the highest concentration of mannoproteins and varietal volatile compounds (terpenes and norisoprenoids). In order to determine the grade of retention of these varietal volatiles by mannoproteins, the colloidal fraction obtained from each wine was precipitated and isolated. Colloids were then added to a model wine and this solution was spiked with a known concentration of 2 aromatic compounds: geraniol and linalool. The results obtained showed that the colloidal fraction from *S. cerevisiae* 1 wine retained the highest percentage of geraniol and linalool compared with the results obtained from the rest of wines tested, including the colloidal fraction from the non-inoculated wine. These results suggest that mannoproteins could be involved in the retention of varietal compounds in the wines elaborated with *S.cerevisiae* 1 contributing to improve its sensorial profile. Most relevant scientific results have been published in the article 2.

4. Effect of short ageing on lees on the mannoprotein content, aromatic profile and sensorial character of white wines.

The interaction of mannoproteins released during the alcoholic fermentation and ageing process, with typical varietal compounds, has been proved in previous chapters of the present thesis. Ageing on lees is a technique frequently used in wineries to improve the

concentration of mannoproteins in wines. In white wines, this practice is often empirically used, although there is no consensus among wine producers about the benefits of its application in wines. The main concerns are based in long wine storage times on lees associated with the reduction of freshness in white wines. For this reason, the aim of the current work was to study the effect of different ageing on lees times on the aromatic profile and sensorial characteristics of Albariño white wines fermented by *S.cerevisiae* 1 strain.

White Albariño wines fermented in winery conditions (30 L) with the selected strain *S. cerevisiae* 1, were aged on lees during different periods of time: 10 days, 20 days, 30 days, 40 days, and 50 days. Additionally, a control wine was prepared without aging on lees. The concentration of polymeric mannose in Albariño reached a maximum at 20 days, decreasing later until more advanced times of aging. Also, most of the varietal aromas identified shown its highest concentration at 20 days of aging on lees. Other compounds such as esters and acetates had a similar behaviour. In the sensorial evaluation, all the wines with aging on lees were better evaluated than the wine without aging and the wine with 20 days of ageing on lees was the best scored. The yeast *S. cerevisiae* 1 produced the wines with the best sensorial character after 20 days of aging on lees. This time is also related with the highest concentration of some key aroma compounds and mannoproteins. Further aging times decreased the sensorial quality of the wine, also modifying its analytical composition in both, aroma compounds and mannoproteins. This ageing on lees process with *S.cerevisiae* 1 strain was protected through the patent "Quality improvement of Albariño wines through anaerobic biological ageing with the ecotipycal *S. cerevisiae* DSM 21378 yeast" (**Annexe 3**). The patent is actually entitled to Terras Gauda winery, which is currently using this method in the production of its wines. Most relevant scientific results are presented in the article 3 of the current thesis.

5. Chemical evaluation of white wines elaborated with a recombinant *Saccharomyces cerevisiae* strain overproducing mannoproteins.

In the two strategies for improving the quality of Albariño wines described earlier in this thesis that have been patented and are used by a wine producer to elaborate its wines, the strain *S. cerevisiae* 1 has been used. One of the most relevant properties of this strain is

its ability to produce wines rich in mannoproteins.

Following this trend, the *S. cerevisiae* strain EKD-13 was selected to carry out the alcoholic fermentation of Albariño grape must. EKD-13 is a recombinant *S.cerevisiae* strain derived from the commercial EC1118 strain, which was engineered for a higher mannoprotein production, and that was previously developed in our laboratory in the Institute for Industrial Fermentations (CSIC), under the supervision of Dr. Ramón González

The recombinant EKD-13 yeast strain has been previously used to obtain white wines from the Sauvignon-Blanc variety enriched in non-specific mannoproteins, that help to protect against protein haze (Gonzalez-Ramos *et al.,* 2008). In the present work, the main purpose was to study the behaviour of this strain in the fermentation of Albariño must and if the mannoproteins released have any impact on the aroma and phenolic compounds in the obtained wines.

Albariño must was fermented with the EKD-13 and the parental strain EC1118. A non-inoculated fermentation was used as experimental control. As expected, the wines fermented with transgenic strain EKD-13 presented the highest concentration of mannoproteins, around 600 mg/L, 4 times higher than the average concentration found in wines. The analysis of the main volatile compounds present showed that the most significant change observed in the wine made with the EKD-13 strain was the increased concentration of 2-phenylethanol. Although we do not know the practical implications of the accumulation of 2-phenylethanol in this specific wine, it is interesting that in the group of higher alcohols this compound is the only one which, if present at higher concentrations, could enhance the aroma of these types of wines, conferring them floral aromatic notes, which could be distinctive in white Albariño wines. Interestingly, in spite of their higher concentration of mannoproteins, an increase of varietal aroma compounds (terpenes and norisoprenoids) was not detected in wines made with the EKD-13 strain, showing that specific mannoproteins related to the yeast strain could be responsible for this behaviour, which does not depend on the final concentration of mannoproteins present in the wine.

Concerning the phenolic composition, the most distinctive character was the high concentration of tyrosol in the wines fermented with EKD-13. The higher content of tyrosol in the wines elaborated with the recombinant EKD-13 strain is consequent with the higher values found for total polyphenols and TPI in these wines, as well as their higher browning

potential. Significant differences among 2-phenylethanol and tyrosol concentration could be due primary to the expression of selection marker genes (ARO4-OFP) in fermentation conditions, which effect in the metabolism of these compounds was described in the article 4 of this thesis where most relevant results of this study are also gathered.

6. Effect of malolactic fermentation by *Pediococcus damnosus* on the composition and sensory profile of Albariño and Caiño white wines.

In contrary to previous chapters in this memory, in the last one, malolactic fermentation (MLF) and lactic bacteria (LAB) were selected as biotechnological tools to improve the quality of Albariño white wines from Rias Baixas PDO. MLF is not a regular process in white wines from this region, since the peculiar acidity of these wines enhances some characteristic aromatic compounds. Nevertheless, homogenizing conditions of production described before and an increasing competence between producers have induced an existing interest in wineries towards the production of wines with distinctive properties through the malolactic fermentation of wines.

For this reason, we performed a study to determinate the influence of MLF on the organoleptic characteristics of Albariño and Caiño wine from Rias Baixas PDO. A commercial LAB strain of *Oenococcus oeni (O.oeni)* was inoculated in Albariño and Caiño white wines to conduct MLF. The results obtained showed that *O. oeni* commercial strain did not dominate the MLF. Two indigenous strains of *Pediococcus damnosus* (*P.damnosus*), C5 and C8, were the main responsible of the FML in Caiño white wines. Both strains of *P.damnosus* were inoculated in Albariño white wines, but only one of them (C5), had conducted the FML. Molecular analysis confirmed the lack of genes implicated in the production of exopolysaccharides and biogenic amines. The sensory analysis showed that FML produced significant changes in some organoleptic descriptors. For example, the colour of Albariño wines got more yellow-golden after MLF and typical fruity odor descriptors (pear and apple), disappeared and a new one, vanilla, appeared in wines. Likewise, the acidity decreased and final wines presented an increase in body and softness. In Caiño wines, the inoculation and fermentation with *P.damnosus caused* the loss of “fresh” notes such as fresh herbs and banana, typical from Caiño wines, an increase the honey descriptor, also presenting a new descriptor: caramel.

This work was a result of a collaboration with the Centro de Ricerca per l' enologia (CRA), which was founded through an European mobility grant BES- 2007- 16606 and the project AGL2006-02558 under the supervision of Dr. Emilia García-Moruno. As results of this last part of the current thesis, our laboratory and CRA patented the use of both *P. damnosus* strains (C5, C8) for the MLF of Albariño and Caiño white wines (**Annexe 4**), patent that was entitled to Terras Gauda winery for its use it in the production of wines. Most relevant results of this study are presented in the article 5 of the current thesis.

CONCLUSIONS

First

The alcoholic fermentation of Albariño grape musts with the authocthonous *Sacharomyces cerevisie* strain 1 (*S.cerevisiae*), significantly affected the volatile and phenolic profile of resulting wines, improving sensorial characteristics. White wines fermented with this strain presented higher concentrations of volatile compounds, mainly terpenes and norsoprenoids, which are related with the typical fruity character and freshness of these wines, and lesser concentration of hidroxycinamic acids and flavan-3-ols associated with bitterness, astringency and browing potential.

Second

Wines fermented with the autochtonous *S.cerevisie* 1 strain showed a higher concentration of both mannoproteins and volatile compounds (terpenes and norisoprenoids), than the rest of wines tested. A study in model wine showed that colloidal fraction of wines fermented with *S.cerevisiae* 1 retained a higher percentaje of linalool and geraniol in comparation with fractions isolated from other wines studied. These results indicated that mannoproteins release from *S. cerevisiae* 1 strain preferentially retain some aromatic compounds.

Third

Short ageing on lees during 20 days of white wines fermented by *S. cerevisie* 1 strain have produced better sensorial quality wines. At this time, wines presented higher concentration of mannoproteins and key aromatic compounds.

Forth

Transgenic *S.cerevisie* EKD-13 strain completely fermented Albariño grape must giving wines with distinct chemical characteristics mainly due to the expression of genes transformed in winemaking conditions. Most significant characteristics incluyed an important increase of mannoproteins, 2 phenyl ethanol and tyrosol concentration. Although some of

these features may be useful in terms of both technological and sensorial scene, a sensorial evaluation is required in the future to asses their effect in the sensorial perception of these wines.

Fifth

Two authocthonus *Pediococcus damnosus* strains (C5 and C8) which were isolated had the capacity to carry out the malolactic fermentation of wines from white Caiño grape variety, being among them one, (C5), able to conduct the malolactic fermentation of Albariño white wines. Selected and isolated strains did not produce biogenic amines (lack of genes hdc, tdc and odc), neither exoplysaccharides (lack of gen dps). Malolactic fermentation significant modified sensorial characteristics giving wines with distint properties from among which stands the decrease of typical herbaceous and fruity descriptors and the increase of colour intensity and honey and vanilla notes.

Primera

La vinificación de mostos Albariño con la cepa autóctona de levadura *Saccharomyces cerevisiae* 1 (*S.cerevisiae*), influyó significativamente en la composición volátil y en el perfil fenólico de los vinos obtenidos, mejorando sus propiedades sensoriales. Los vinos blancos obtenidos con esta cepa tuvieron una mayor concentración de compuestos volátiles, principalmente terpenos y norisoprenoides, relacionados con el carácter frutal y la frescura de estos vinos, y una menor concentración de ácidos hidroxicinámicos y flavan 3-oles, relacionados con la astringencia, el amargor, y el índice de pardeamiento.

Segunda

Los vinos fermentados con la cepa autóctona *S.cerevisiae* 1 presentaron una mayor concentración de manoproteínas y de compuestos volátiles (terpenos y norisoprenoides), que el resto de vinos analizados. En experimentos realizados en medio vínico modelo, se demostró que la fracción coloidal del vino elaborado con la cepa *S. cerevisiae* 1 retuvo un porcentaje de geraniol y linalool mayor que la fracción coloidal del resto de los vinos, indicando que las manoproteínas liberadas por esta cepa pueden retener preferencialmente algunos compuestos del aroma.

Tercera

La crianza sobre lías por un período de 20 días de los vinos blancos elaborados con la cepa *S. cerevisiae* 1 dió lugar a vinos de mejor calidad sensorial. En este tiempo de crianza sobre lías los vinos presentaron también una mayor concentración de manoproteínas y de compuestos claves del aroma.

Cuarta

La cepa de levadura transgénica *S.cerevisiae* EKD-13 fermentó completamente el mosto Albariño produciendo vinos con algunas características químicas distintivas en su mayor parte derivadas de la expresión de los genes modificados en las condiciones de la

fermentación. Las más significativas fueron el incremento de la concentración de manoproteínas, del compuesto aromático 2-fenil etanol y del compuesto fenólico tirosol. Aunque algunas de estas características podrían ser útiles tanto desde el punto de vista tecnológico como sensorial, se requiere evaluar en el futuro el impacto de estas modificaciones en la percepción sensorial global de los vinos obtenidos.

Quinta

Se aislaron dos cepas de *Pediococcus damnosus* (C5 y C8) capaces de realizar la fermentación maloláctica en los vinos elaborados con la variedad Caiño Blanco, siendo una de ellas (C5) capaz de llevar a cabo la fermentación maloláctica en vinos de la variedad Albariño. Las cepas aisladas no producen aminas biógenas (ausencia de genes hdc, tdc y odc) ni exopolisacáridos (ausencia del gen dps). La fermentación maloláctica modificó de forma significativa las propiedades sensoriales de estos vinos, obteniéndose unos vinos con unas propiedades características, en los que se puede destacar la disminución de los descriptores típicos herbáceos y frutales y el aumento de la intensidad del color y de los descriptores sensoriales relacionados con la miel y la vainilla.

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ANNEXES

Annexe 2: Patent 1; " A process to obtain wine from Albariño white varieties (and others) with high aromatic content with a ecotipic yeast "

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(54) Título: **Procedimiento para obtener vinos de la variedad de uva Albariño (y otras) con alto contenido aromático mediante el uso de una levadura ecotípica.**

(57) Resumen:
Procedimiento para obtener vinos de la variedad de uva Albariño (y otras) con alto contenido aromático mediante el uso de una levadura ecotípica.
Microorganismo de la especie *Saccharomyces cerevisae* capaz de inducir un alto nivel de terpenos volátiles además del uso de los mismos para la elaboración de vinos a partir de mosto de uva, de cualquier fruta o de cualquier solución hidroazucarada.

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Annexe 3: Patent 2; "Quality improvement of Albariño wines through anaerobic biological ageing with the ecotipycal *S. cerevisiae* DSM 21378 yeast"

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(54) Título: **MEJORA DE LA CALIDAD DE VINOS DE LA VARIEDAD ALBARIÑO MEDIANTE CRIANZA BIOLÓGICA ANAEROBIA CON LA LEVADURA ECOTÍPICA SACCHAROMYCES CEREVISAE DSM 21378.**

(57) Resumen:
Mejora de la calidad de vinos de la variedad Albariño mediante crianza biológica anaerobia con la levadura ecotípica Saccharomyces cerevisae DSM 21378.
La presente invención se refiere al uso del microorganismo de la especie Saccharomyces cerevisae DSM 21378 o de una micropoblación que comprende dicho microorganismo, para la crianza biológica anaerobia de vino producido con uvas de la especie Vitis vinifera var. Albariño. Dicha cepa DSM 21378 o una población que la comprende, pueden usarse para el aumento de coloides glicoproteicos en dicho vino, donde preferiblemente dichos coloides glicoproteicos son manoproteínas, aunque pueden ser también de naturaleza polisacarídica, y/o para aumentar los componentes aromáticos.

ES 2 376 206 B1

Annexe 4: Patent 3; "A process to carry out the malolactic fermentation of wines from Caiño and Albariño white varieties (*Vitis vinífera* L.) with Pediococcus damnosus DSM 25074 and DSM 25075"

MINISTERIO DE INDUSTRIA, TURISMO Y COMERCIO

Oficina Española de Patentes y Marcas

Justificante de presentación electrónica de solicitud de patente

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica, utilizando la conexión segura de la O.E.P.M. Asimismo, se le ha asignado de forma automática un número de solicitud y una fecha de recepción, conforme al artículo 14.3 del Reglamento para la ejecución de la Ley 11/1986, de 20 de marzo, de Patentes. La fecha de presentación de la solicitud de acuerdo con el art. 22 de la Ley de Patentes, le será comunicada posteriormente.

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Solicitante:	CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS (CSIC) 50%	
Número de solicitantes:	2	
País:	ES	
Título:	UN PROCEDIMIENTO PARA LA REALIZACIÓN DE LA FERMENTACIÓN MALOLÁCTICA EN VINOS PROVENIENTES DE LAS VARIEDADES ALBARIÑO Y CAIÑO BLANCO (VITIS VINIFERA, L.) CON PEDIOCOCCUS DAMNOSUS DSM 25074 Y DSM 25075	
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MINISTERIO
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(1) MODALIDAD: **PATENTE DE INVENCIÓN** **MODELO DE UTILIDAD**	 [✓] [ ]
(2) TIPO DE SOLICITUD: PRIMERA PRESENTACIÓN ADICIÓN A LA PATENTE EUROPEA ADICIÓN A LA PATENTE ESPAÑOLA SOLICITUD DIVISIONAL CAMBIO DE MODALIDAD TRANSFORMACIÓN SOLICITUD PATENTE EUROPEA PCT: ENTRADA FASE NACIONAL	 [✓] [ ] [ ] [ ] [ ] [ ] [ ]
(3) EXP. PRINCIPAL O DE ORIGEN: MODALIDAD: N.º SOLICITUD: FECHA SOLICITUD:	
4) LUGAR DE PRESENTACIÓN:	OEPM, Presentación Electrónica
(5) DIRECCIÓN ELECTRÓNICA HABILITADA (DEH):	
(6-1) SOLICITANTE 1: DENOMINACIÓN SOCIAL: NACIONALIDAD: CÓDIGO PAÍS: DNI/CIF/PASAPORTE: CNAE: PYME: DOMICILIO: LOCALIDAD: PROVINCIA: CÓDIGO POSTAL: PAÍS RESIDENCIA: CÓDIGO PAÍS: TELÉFONO: FAX: PERSONA DE CONTACTO: MODO DE OBTENCIÓN DEL DERECHO: INVENCIÓN LABORAL: CONTRATO: SUCESIÓN:	 CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS (CSIC) 50% España ES Q 2818002D SERRANO, 117 MADRID 28 Madrid 28006 España ES [✓] [ ] [ ]
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(8) TÍTULO DE LA INVENCIÓN:	UN PROCEDIMIENTO PARA LA REALIZACIÓN DE LA FERMENTACIÓN MALOLÁCTICA EN VINOS PROVENIENTES DE LAS VARIEDADES ALBARIÑO Y CAIÑO BLANCO (VITIS VINIFERA, L.) CON PEDIOCOCCUS DAMNOSUS DSM 25074 Y DSM 25075
(9) PETICIÓN DE INFORME SOBRE EL ESTADO DE LA TÉCNICA:	
SI	[ ]
NO	[✓]
(10) SOLICITA LA INCLUSIÓN EN EL PROCEDIMIENTO ACELERADO DE CONCESIÓN	
SI	[ ]
NO	[✓]

(11) EFECTUADO DEPÓSITO DE MATERÍA BIOLÓGICA:	
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NO	[ ]
(12-1) DEPOSITO 1:	
REFERENCIA DE IDENTIFICACIÓN:	DSM25074
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NÚMERO DE DEPÓSITO:	DSM25074
ACCESIBILIDAD RESTRINGIDA A UN EXPERTO (ART. 45.1. B):	[✓]
(12-2) DEPÓSITO 2:	
REFERENCIA DE IDENTIFICACIÓN:	DSM25075
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NÚMERO DE DEPÓSITO:	DSM25075
ACCESIBILIDAD RESTRINGIDA A UN EXPERTO (ART. 45.1. B):	[✓]
(13) DECLARACIONES RELATIVAS A LA LISTA DE SECUENCIAS:	
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LA LISTA DE SECUENCIAS EN FORMATO PDF Y ASCII SON IDENTICOS	[ ]
(14) EXPOSICIONES OFICIALES:	
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(16) AGENTE/REPRESENTANTE:	
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